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CONCERNING THE ORIGIN OF BACTERIAL VIRUSES

By John H. Northrop

LABORATORY OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH, DEPARTMENT OF BACTERIOLOGY, UNIVERSITY OF CALIFORNIA, BERKELEY

Communicated January 8, 1958

Introduction.—The essential characteristics of bacterial viruses (bacteriophages) are now pretty well established. They are nucleoproteins,[1] produced by the bacterial cell,[2] which are capable of transmitting genetic information from one bacterium to another.[3] The information is carried by the nucleic acid.[4] The virus may therefore be considered a specialized type of transforming principle. Many, if not all, bacterial species produce them. The production of the virus is a genetic property of the host cell.[5] All the cells of a lysogenic culture are potentially capable of producing virus, but only a few contain virus particles.[6] The remaining cells contain no virus and no virus antigen,[7] so that the virus has no continuous existence and part of the time is indistinguishable from the host cell.

The virus has no metabolism[8] and is unable to use energy to synthesize itself; according to the writer's definition,[9] therefore, it is not living. If it is living, then the reactivation of inactive virus[10] is tantamount to the creation of life.

The origin of the virus and its characteristic properties may be accounted for by the assumption that the virus is the result of a mutation of the bacterial cell.* After the mutation occurs, the cell produces virus particles instead of more cells. These particles are able to transmit the property of producing virus to other cells. They may be considered, therefore, as "hereditary lethal mutants." The new cell may then occasionally back-mutate, to give a new lysogenic cell similar to the original one. This process automatically prunes off any phage-sensitive mutants which may arise, by destroying them or turning them back into phage-resistant lysogenic cells like the original culture.

If this assumption is correct, it would be expected that the proportion of phage-producing cells would be increased by mutagenic agents, and Lwoff and his collaborators[12] have shown that this is the case.

It would also be expected that other mutants present in the culture would be increased to about the same extent as the phage-producing cells, since mutagenic agents in general increase the proportion of all mutants.[13]

The rate of appearance of the virus should be predictable by the same equations which predict the rate of appearance of a known mutant. In order to test these predictions, the effect of ultraviolet light and hydrogen peroxide on the incidence of phage-producing cells and of terramycin-resistant cells (a proved mutation)[14]

TABLE 1*

EFFECT OF ULTRAVIOLET LIGHT ON PROPORTION OF TERRAMYCIN-RESISTANT CELLS AND OF VIRUS IN VARIOUS STRAINS OF *B. Megatherium*

B. megatherium	Culture Medium	Ultraviolet Light Distance (Cm.)	Ultraviolet Light Time (Min.)	TR/Cell × 10^8	P/Cell × 10^8	Mutation Rates by Null Fraction TR	Mutation Rates by Null Fraction P
899	YEP (yeast-extract peptone)	...	0	15	0.5×10^8	...	...
899	YEP (yeast-extract peptone)	10	5	20	20×10^8	...	...
899	YEP (yeast-extract peptone)	50 2-mm. slit	Continuous	20	3×10^8	...	...
899	YEP (yeast-extract peptone)	1	2	...	Lysis, $> 1 \times 10^{10}$	...	...
899SP	YEP (yeast-extract peptone)		0	3.5×10^3	1.5×10^6	...	...
		5	10	5.6×10^3	50×10^6	...	...
899Y	YEP (yeast-extract peptone)		0	20	1×10^7	6×10^{-8}	7×10^{-4}
		5	10	20	1×10^7	7×10^{-8}	15×10^{-4}

* The figures are averages of six to twenty separate experiments. The average deviation of the mean is about ±20 per cent in most cases. The ultraviolet-light experiments were carried out nearly a year before those with hydrogen peroxide, during which time the strains changed somewhat. Terramycin-resistant cells (TR) and virus (P) were determined as previously described (J. H. Northrop, *J. Gen. Physiol.*, **40**, 547, 1957). The mutation rates were determined by the null fraction method (see J. Lederberg, in *Bacterial Physiology*, ed. C. H. Werkman and P. W. Wilson [New York: Academic Press, Inc., 1951]; and J. H. Northrop, *J. Gen. Physiol.*, **41**, 131, 1957).[13, 15] A General Electric germicidal lamp was used.

TABLE 2

EFFECT OF HYDROGEN PEROXIDE ON PROPORTION OF TERRAMYCIN-RESISTANT CELLS AND OF VIRUS IN VARIOUS STRAINS OF *B. megatherium*

B. megatherium	Culture Medium	Hydrogen Peroxide (Mol/l)	TR/Cell × 10^8	P/Cell × 10^8	Mutation Rates by Null Fraction TR	Mutation Rates by Null Fraction P
899	YEP	0	50	3×10^8	2.5×10^{-8}	6×10^{-3}
		1×10^{-5}	80	8×10^8	...	...
		5×10^{-5}		$>1 \times 10^{12}$ Lysis	...	...
899	Arginine Aspartic Acid	0	6	3×10^8	3×10^{-8}	1.2×10^{-2}
		3×10^{-5}	10	8×10^8	5×10^{-8}	20×10^{-2}
		1×10^{-4}	25	20×10^8 (some lysis)	...	...
899	Ammonium sulfate	0	7	2.3×10^6	...	1×10^{-4}
		1×10^{-4}	30	13×10^6	...	...
899Y	YEP	0	26	4×10^6	4×10^{-8}	1.6×10^{-4}
		1×10^{-4}	70	14×10^6	18×10^{-8}	5.0×10^{-4}
899Y	Ammonium sulfate	0	15	3×10^3	2×10^{-8}	3×10^{-6}
		1×10^{-4}	30	10×10^3	6×10^{-8}	7×10^{-6}
899Y1	YEP	0	<1	4×10^6	...	...
		3×10^{-5}	<1	10×10^6	...	...
KM	YEP	0	5	$<1 \times 10^{-2}$	...	...
		5×10^{-5}	30	$<1 \times 10^{-2}$	...	...

in *B. megatherium* cultures has been determined. A brief summary of some of these experiments is given in Tables 1 and 2.

Ultraviolet light increases the proportion both of terramycin-resistant mutants and of virus in *megatherium 899* cultures in yeast-extract peptone. The increase in the proportion of the two mutants is about the same at low intensities, but higher intensities cause the culture to change completely to the virus (lysis) (cf. p. 234).

The number of terramycin-resistant cells cannot be determined in such cultures, since no colonies are formed.

The SP strain produces more terramycin-resistant cells and also more phage after exposure to ultraviolet light. In this case also the increase in phage is greater than the increase in terramycin-resistant cells with high doses of ultraviolet light.

No change in either ratio occurs when a yellow mutant, *899Y*, is exposed to low intensities of ultraviolet light. Long exposure sometimes results in lysis.

Growth of *899* or *899Y* in low concentrations of hydrogen peroxide in either yeast-extract peptone, asparagine-aspartic acid culture medium, or ammonium sulfate culture medium results in a three- to fourfold increase in both terramycin-resistant mutants and virus. No lysis occurs in ammonium sulfate culture medium. Higher peroxide concentrations cause lysis in yeast-extract peptone.

The mutation rates determined by the null fraction method show that the increase in virus and terramycin-resistant cells is due to an increase in their respective mutation rates and not simply to a change in the growth rates or in the number of virus particles produced per cell.

A mutant clone of *899Y*, *Y1*, produced no detectable (i.e., <1) terramycin-resistant colonies per 10^8 cells for some time. During this time, no terramycin-resistant colonies could be induced by either ultraviolet light or hydrogen peroxide.

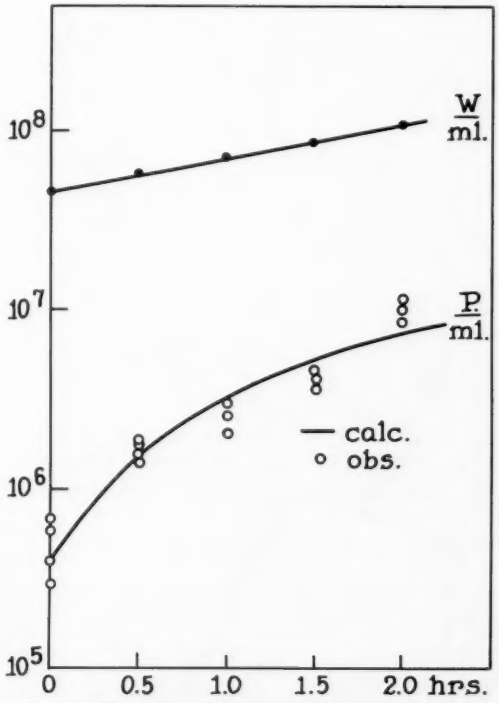

FIG. 1.—Phage (P)/ml and cells (W)/ml in a culture of *megatherium 899* in yeast-extract peptone at 20°. Logarithmic growth *899* centrifuged and washed twice in yeast-extract peptone and shaken at 20°. Three phage determinations on each sample. Results calculated by equation (4). $l = 50$, $C = 0.8 \times 10^{-3}$, $W_0 = 5 \times 10^7$, $P_0 = 4 \times 10^5$, $A = 0.44$.

The sensitive strain of *B. megatherium* (*KM*) produces no detectable ($<1 \times 10^{-2}$) virus particles per 10^8 cells, and so far all attempts to produce virus in this strain have failed.

If the culture contains <1 per cent of the number of mutants which can be detected, then treatment with ultraviolet light or hydrogen peroxide would not be expected to cause a mutant to appear, since the mutation rates with *B. megatherium* in general do not increase much more than a hundred times. There are many re-

ports in the older literature of the "production" of bacterial virus by treatment with various substances, usually mutagens. Similar experiments have recently been reported by Herčík[17] and Hradečná.[18] Yamafuji[19] has described the production of polyhedral virus in silkworms by various reagents. Such results are to be expected, if the virus is a result of a cell mutation. A virus disease, therefore, may be infectious and also endogenous.

Calculation of the Concentration of Virus.—If the phage-producing cells are mutants, then the number of these cells, formed at various times, should be in agreement with the equations[20] which describe the occurrence of terramycin-resistant mutants in *B. megatherium* cultures.[16] The following experiments show that this is true.

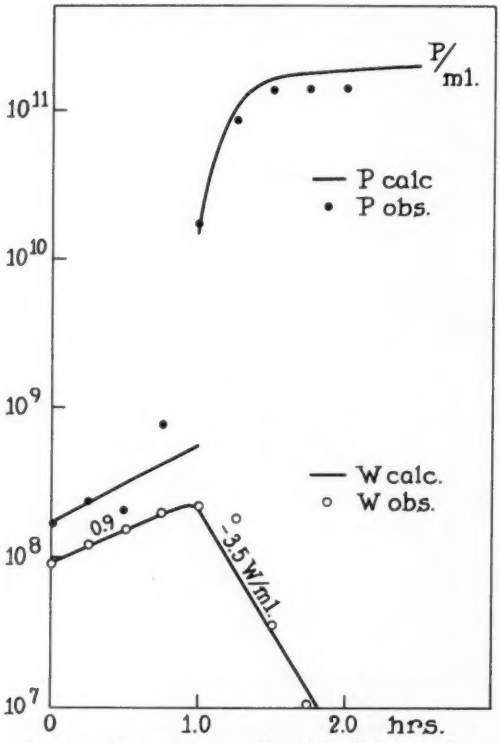

FIG. 2.—Effect of strong ultraviolet light on phage (*P*) and cell concentration (*W*). *Megatherium 899* in logarithmic growth in yeast-extract peptone at 35° exposed to General Electric utraviolet light for 10 minutes at 10 cm. distance and then shaken at 35°. Results calculated from equation (4). $l = 700$, $C = 4.4$, $A = 0.9$, $W_0 = 2 \times 10^8$, $P_0 = 1.5 \times 10^{10}$, t = (elapsed time − 1.0). The results are calculated *as if* the phage were liberated as soon as the mutation occurred.

Calculation of the Number of Virus Particles.—If the mutation occurs without cell division, the number of mutants and cells present at various times is[20]

$$M = \frac{CW_0[e^{(A-C)t} - e^{Bt}]}{A - B - C} + M_0 e^{Bt}, \quad (1)$$

$$\frac{M}{W} = \frac{C[e^{(B+C-A)t} - 1]}{B + C - A} + \frac{M_0 e^{(B+C-A)t}}{W_0}, \quad (2)$$

$$W = W_0 e^{(A-C)t}, \quad (3)$$

in which W is the concentration of wild cells, M is the concentration of mutants, C is the mutation time rate constant, A is the growth rate constant of W, and B is the growth rate constant of M. If $C << A$, $C = 2\lambda A$, where λ is the mutation frequency rate constant. In this case the equations are identical with those derived by means of the assumption that mutants appear only after cell division.

If $C > A$, however, the equations for the case in which mutants appear as a constant fraction of the new cells are quite different from those in which the mutants

appear as a constant fraction of the cells per unit of time.

In the case of phage-producing cells, $M = P/l$, where P is the phage concentration, l is the number of phage particles released per cell, and $B = 0$, since the phage-producing cells do not multiply. Equation (1) then becomes

$$P = \frac{lCW_0[e^{(A-C)t} - 1]}{A - C} + P_0, \quad (4)$$

and equation (2) becomes

$$\frac{P}{W} = \frac{lC[e^{(C-A)t} - 1]}{C - A} + \frac{P_0 e^{(C-A)t}}{W_0}. \quad (5)$$

If $C << A$,

$$\frac{P}{W} \rightarrow \frac{lC}{A} \quad \text{as} \quad t \rightarrow \infty.$$

If $C > A$,

$$\frac{P}{W} \rightarrow \infty \quad \text{as} \quad t \rightarrow \infty.$$

The rate of appearance of virus in *B. megatherium 899* cultures in yeast-extract peptone is shown in Figure 1. The rate of appearance of the virus after exposure of the culture to ultraviolet light is shown in Figure 2. The solid lines are calculated by equation (4). In both experiments the calculated results agree quite well with the observed figures. The values of the constants used are close to the values determined by independent methods. The rate of lysis of the cells, after exposure to ultraviolet light, is greater than the growth rate of the culture. The change (mutation) which leads to lysis, therefore, must occur without cell division, since otherwise it is impossible for the cells to change at a rate faster than the growth rate.[20]

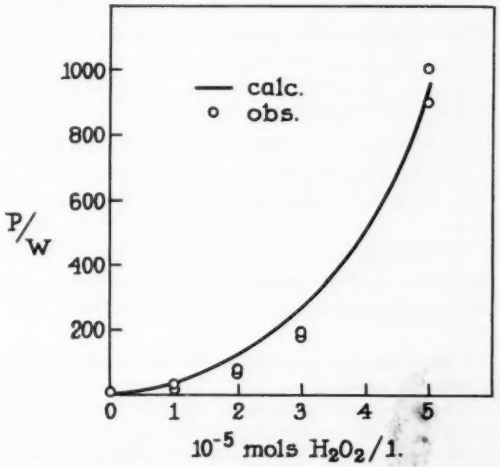

FIG. 3.—Ratio of virus particles to cells (P/W) of *megatherium 899* in yeast-extract peptone containing various concentrations of hydrogen peroxide after 2 hours' growth at 35° C. Results calculated from equation (5). $t = 2$, $l = 100$, $C = 0.1 + (3 \times 10^4) \times$ (mols H_2O_2/l).

H_2O_2 mols/l.....	0	1	2	3	5×10^{-5}
A.....	2	1.6	1	0.7	0.5

The effect of various concentrations of hydrogen peroxide on the ratio of virus to cells is shown in Figure 3. In this case it is assumed that C, the mutation time rate constant, increases in proportion to the hydrogen peroxide concentration. This is the usual relationship for low concentrations of a mutagenic agent and leads to results which agree quite well with the experiment. This experiment was carried out for 2 hours. If the experiment is continued much longer, the cultures return to normal growth, because of the decomposition of the hydrogen peroxide. The results show that, as long as the mutation rate C is small compared to the growth rate A, small increases in C result in proportionate increases in the ratio of P/W. As C approaches A, however, small increases in C result in great increases in the ratio, and if $C > A$, the ratio approaches infinity.

This relation between mutation rate and growth rate explains the fact that cultures which have a very low mutation rate compared to the growth rate (for instance, *megatherium 899* in ammonium sulfate culture medium) will not be caused to lyse by increasing the mutation rate ten to twenty times, while cultures which have a higher mutation rate will lyse completely.

A detailed report of these experiments will be published in another journal.

Summary.—The effect of ultraviolet light and hydrogen peroxide on the incidence of terramycin-resistant mutants and of phage-producing cells in *B. megatherium* cultures has been determined.

The incidence of the two types of cells is increased by both ultraviolet light and hydrogen peroxide. The increase is due to an increase in the rate of incidence (mutation rate) and not to changes in the growth rate, or yield of virus.

The rate of appearance of the phage-producing cells is in agreement with the same equations which predict the rate of appearance of terramycin-resistant mutants.

The change from a normal to a terramycin-resistant cell has been shown to be a mutation, and the present results therefore suggest that the change from a normal to a phage-producing cell is also a mutation.

* Since the virus is indistinguishable from the host cell at the time the mutation occurs, it is a matter of convention whether the mutation is ascribed to the virus or the cell.[11] The facts are the same as in the appearance of a new enzyme in a cell-enzyme system. This phenomenon is conventionally considered to be the result of a cell mutation; the principle of economy of hypothesis favors a similar convention in respect to a mutation which occurs in a cell-virus system. There is some direct evidence concerning the origin of the mutation. A single cell produces one type of virus and not a mixture. If the virus mutates as it multiplies, a mixture of virus particles would result, just as in the case of bacterial mutants (Jacob).[11] This result was confirmed during the present work.

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*DEHYDRATED MUSCLE FIBERS**

BY HELMUT MUELLER†

INSTITUTE FOR MUSCLE RESEARCH AT THE MARINE BIOLOGICAL LABORATORY, WOODS HOLE, MASSACHUSETTS

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Myosin denatures on freeze-drying, and so it seemed impossible to dry muscle without destroying its contractility. Having found[1] that a dry contractile muscle could be obtained by dehydrating with acetone at a low temperature, I tried to freeze dry muscle and obtained a preparation which contains only traces of water and contracts on rehydration, developing tensions comparable to those developed by the same muscle in vivo. This preparation can be made within several hours.

After completion of this work, I learned that similar procedures had already been successfully employed by Hopkins.[2, 3] Since my experience is somewhat different and extends to different points, a brief presentation seemed indicated.

Strips from rabbit psoas muscle were tied to applicator sticks, as in the glycerination procedure.[4] They were rapidly frozen in powdered dry ice, transferred to a lyophilizer, and evacuated for 16–18 hours. The dried muscle is white and appears feather-light. There is no shrinkage associated with the loss of water. With a needle, thin fiber bundles of any desired diameter can be separated. Although most of the subsequent experiments were performed with strips 0.1–0.3 mm. in diameter, much thinner bundles can also be isolated. These are supple and flexible but not extensible. The water content was 5–8 per cent and diminished to 1–2 per cent in the desiccator over P_2O_5.

When these fibers were placed in water, they contracted, first very rapidly, then more slowly, reaching their maximal shortening (about 80 per cent) in often less than 1 minute. Addition of ATP to the contraction bath caused a slow further

shortening, up to 90 per cent (Fig. 1). The contraction of lyophilized fibers requires only the addition of water, since their ATP is preserved. They will not contract, however, in any solvent other than water. Like fresh muscle fibers, the lyophilized ones may be extracted with 50 per cent glycerol at $-20°$ C. and will then require the addition of ATP for contraction. Though they contract more slowly, with sufficiently high ATP concentrations the same degree of maximal shortening is reached (Fig. 1).

It is also possible to perform the opposite experiment, namely, to freeze dry glycerol-extracted fibers, after washing out the glycerol. They will also contract slowly on addition of water, ATP, and ions.

On isometric contraction of lyophilized fibers immersed in water or diluted salt solutions, a rapid rise in tension can be recorded. This can be demonstrated by

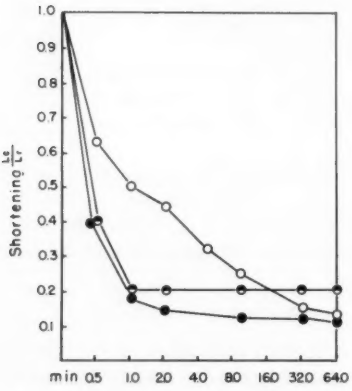

FIG. 1.—Shortening of lyophilized muscle fibers. Bath contained 0.1 KCl and 4 mM $MgCl_2$. *Half-circles:* no ATP added; *full circles:* 6 mM ATP; *empty circles:* fibers glycerol-extracted for 4 weeks after lyophilization in presence of 6 mM ATP. $\frac{Lc}{Lr}$ = length of contracted fiber / length of uncontracted fiber.

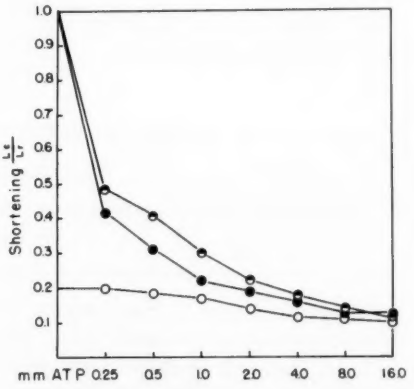

FIG. 2.—Maximal shortening at varied ATP concentration. Contraction bath contained 0.1 KCl and 4 mM $MgCl_2$. *Half-circles:* glycerinated fibers not lyophilized; *full circles:* fibers glycerol-extracted for 4 weeks after lyophilization; *empty circles:* lyophilized fibers.

gluing small cotton loops to the ends of the dried fibers and fastening the latter to a kymograph. From the weight of the fibers, their diameter can be calculated, and the tension developed expressed in kilograms per square centimeter of fresh muscle. The values obtained vary considerably; tensions above 3 kg/cm² have been recorded in some experiments (Hopkins reported a maximal tension of 1.2 kg/cm²).

In fibers with optimal performance, the addition of ATP to the bath brings no further increase in tension, but it increases isotonic shortening. A comparison with Weber's[5] results on the ATP-dependence of isometric contraction and of Blum's[6] on the ATP-dependence of isotonic shortening shows that this same relationship holds for glycerol-extracted fibers. While there is an optimal ATP concentration beyond which tension diminishes, maximal shortening increases as a steady function of rising ATP concentration (Fig. 2).

Tension, in lyophilized fibers, develops somewhat faster in pure water than in 0.1 *M* KCl. Addition of small amounts of Mg^{++} or Ca^{++} have no effect.

Lyophilized fibers were kept in the deep freeze at −20° C. for 3 months without appreciable loss of contractility. Exposure to room air leads to loss of contractility within several days. However, if placed in closed tubes immediately after lyophilization, the dried muscle can be kept at room temperature for several weeks without marked loss of contractility.

The relaxing effect of EDTA on glycerinated fibers was described by Watanabe.[7] In the presence of 6 mM ATP, 0.1 *M* KCl, and 4 mM $MgCl_2$, isometrically contracted lyophilized fibers can also be completely relaxed with 5 mM EDTA (Fig. 3). The relaxing action of EDTA depends on the presence of ATP and ions. Relaxation is somewhat slower than in glycerinated fibers but can be promoted by raising the ATP concentration. Addition of 1 mM $CaCl_2$ to this bath elicits full contraction (Fig. 3).

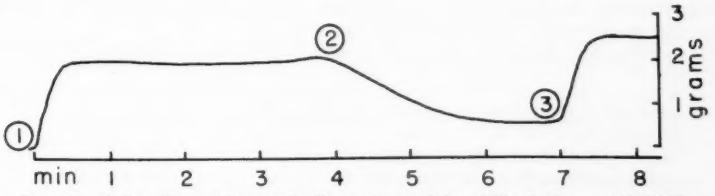

FIG. 3.—Relaxation of isometrically contracted lyophilized fibers with EDTA. At *1*, the preparation was immersed in water; at *2*, 5 mM EDTA, 6 mM ATP, 0.1 *M* KCl, 4 mM $MgCl_2$; and, at *3*, 1 mM $CaCl_2$ were added.

Fibers completely lyophilized in the way described could be relaxed with neither creatine phosphate[8] nor myokinase (Marsh-Bendall Factor).[9]

When, however, the time of lyophilization was reduced to a minimum in order to obtain just macroscopically dry fibers (about 90 minutes), a partial relaxation could be achieved with both factors. The fibers, which still contain 10–15 per cent

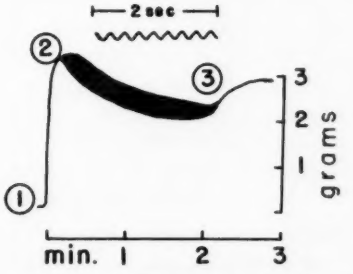

FIG. 4.—Oscillation and relaxation of briefly lyophilized fibers with myokinase. Fibers lyophilized for 90 minutes. Water content = 14 per cent. At *1*, the preparation was immersed in water; at *2*, myokinase, 6 mM ATP, 0.1 *M* KCl, 4 mM $MgCl_2$ were added; at *3*, 1 mM $CaCl_2$. Top shows recording of oscillations with fast-speed kymograph.

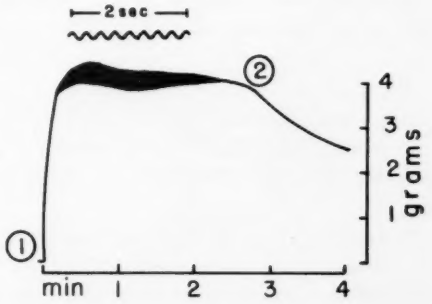

FIG. 5.—Oscillation during maximal tension. Fibers lyophilized for 90 minutes. Water content = 14 per cent. *1:* preparation immersed in water; *2:* 10 mM creatine phosphate, 6 mM ATP, 0.1 *M* KCl, 4 mM $MgCl_2$. Top shows recording of oscillations with fast-speed kymograph.

water, had to be used soon after preparation. The degree of relaxation usually did not exceed 30–40 per cent of the maximal tension. Similar results were obtained with myokinase. These observations support the recent suggestion[10] that relaxation requires a factor in addition to the phosphorylating mechanism. This additional factor appears to be sensitive to dehydration.

I have also observed in our partially dehydrated fibers the auto-oscillation discovered by Goodall and Andrew Szent-Györgyi,[11,12] though I could not reproduce it consistently. I observed it when shortly lyophilized fibers were relaxed or contracted in a bath containing creatine phosphate or myokinase. While in some preparations this occurred almost consistently, it was absent altogether in others. In some instances it even occurred when the fibers were contracted in pure water. Figures 4 and 5 show representative tracings.

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*PTERIDINES IN PHOTOSYNTHESIS**

By R. C. Fuller, I. C. Anderson, and H. A. Nathan†

DEPARTMENT OF BIOLOGY, BROOKHAVEN NATIONAL LABORATORY, UPTON, NEW YORK, AND THE HASKINS LABORATORIES, NEW YORK CITY, NEW YORK

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The chrysomonad, *Ochromonas malhamensis*, has been shown to produce at least two pteridines. Chromatographically one is identical with Citrovorum Factor, a conjugated pteridine; another with Biopterin, an unconjugated pteridine.[1, 2] The present study shows that another chrysomonad, *O. danica;* the green alga, *Chlorella pyrenoidosa;* the euglenid, *Euglena gracilis* Z strain; the photosynthetic bacterium, *Chromatium* D strain; and the duckweeds, *Spriodela oligorrhiza* and *Wolffia punctata* No. 6592, like *O. malhamensis*, each produce at least one conjugated and one unconjugated pteridine. Although some of these pteridines are growth factors for other organisms[1, 2] the functions of these pteridines in the organisms which produce them remained obscure until it was shown that pteridines are intimately involved in the maintenance of photosynthesis in the blue-green alga, *Anacystis nidulans.*[3] When the pteridines were leached from this organism, photosynthesis declined. One pteridine, designated Compound C and subsequently shown to be composed of Biopterin[3] bound to glucose,[4] was found to be derived from $C^{14}O_2$ in fairly long-term fixation experiments with this organism.

That a pteridine may be involved in the early stages of CO_2 fixation in *Chlorella* and *Scenedesmus* was indicated by Dr. Helmut Metzner,[5] who reported that after a 5-second experiment with $C^{14}O_2$ a conjugated pteridine containing C^{14} was obtained. This pteridine was labile after isolation but could be preserved when the $C^{14}O_2$ fixations were terminated with hydroxylamine followed by cold ethanol, a method reported recently by Metzner *et al.*[6]

The present report describes the isolation of C^{14}-labeled pteridines from *Chromatium* sp., *E. gracilis*, *O. danica*, *C. pyrenoidosa*, and *W. punctata*[7] after short-term photosynthetic C^{14}-fixations, using both a modification of the trapping method of Metzner *et al.*[6] and a different trapping agent.

Materials and Methods.—The algae used in these experiments were grown in continuous culture in an atmosphere of 5 per cent CO_2 and under constant illumination. The media for *E. gracilis*, *O. danica*, and *W. punctata* are given in Table 1. *Chlorella pyrenoidosa* was grown in the medium of Myers;[8] *Chromatium* was grown as described by Hendley.[9]

To isolate pteridines from *E. gracilis*, the packed cells were suspended in 0.001 *M* KH_2PO_4 and subjected to sonic oscillation for 10 minutes in a 10-kc Raytheon oscillator. Unruptured cells and debris were collected by centrifugation at 40,000 r.p.m. for 30 minutes. The yellow opalescent supernatant fluid was exhaustively treated with activated charcoal until all the material fluorescing at 3660 A had been adsorbed. The charcoal was then eluted with 50 per cent ammoniacal acetone or 50 per cent ammoniacal ethanol. This eluate was chromatographed on Whatman No. 3 millimeter paper, using butanol:propionic acid.[10] Pteridine spots on the

TABLE 1

MEDIA FOR ALGAE AND DUCKWEED (CONCENTRATION/100 ML)

Material	*E. gracilis*	*O. danica*	*W. punctata*
Nitrilotriacetic acid	0.02 g.	0.02 g.	...
KH_2PO_4	0.03 g.	0.03 g.	0.03 g.
$MgSO_4 \cdot 7H_2O$	0.05 g.	0.1 g.	0.05 g.
$CaCO_3$	0.006 g.	0.005 g.	0.02 g.
EDTA	...	...	0.04 g.
NH_4NO_3	...	...	0.02 g.
NH_4Cl	...	0.05 g.	...
$(NH_4)_2$ citrate	0.2 g.	...	...
Metals 45[14]	2.0 mg.	1.0 mg.	0.8 mg.
$MgCO_3$ (basic)	...	0.04 g.	...
Citric acid $\cdot H_2O$	0.05 g.	...	...
Thiamine $\cdot$ HCl	0.06 mg.	0.1 mg.	...
Biotin	...	1.0 μg.	...
B_{12}	0.5 μg.	...	...
Sucrose	...	...	1.0 g.
Phytone	...	...	0.1 g.
Agar	...	...	0.8 g.
pH	3.5	5.0	5.5

TABLE 2

EFFECT OF TRAPPING AND FIXATION TERMINATION VARIATIONS ON APPEARANCE OF PTERIDINE INTERMEDIATE

Organism Used*	Temp. of Ethanol for Expt. Termination (° C.)	Trapping Agent	Time Added	Appearance of Yellow-fluorescing Pteridine on Chromatograms
E, C, Chr.	+80	None	...	−†
E	+80	NH_2OH	Zero time‡	+
E, C, O, W, Chr.	−40	None	...	−
E, C, O, Chr.	−40	NH_2OH	"End"§	±
E, C, O, W, Chr.	−40	NH_2OH	Zero time	+
E, O, Chr.	−40	Phenylhydrazine	Zero time	+

* *Key:* E = *E. gracilis;* C = *C. pyrenoidosa;* O = *O. danica;* W = *W. punctata;* Chr. = *Chromatium* sp.

† + = always found; ± = small amounts only occasionally found; − = never found.

‡ Trapping agent and $C^{14}O_2$ were added simultaneously.

§ The directions of Metzner *et al.*[9] were followed which allowed contact of NH_2OH for 10–40% of the actual fixation period.

paper were located by scanning for fluorescence with light of about 3660 A wave length.

All algal and bacterial $C^{14}O_2$ fixations were done with 2.0 per cent cell suspensions in a 15 ml. "lollipop" apparatus, as described by Bassham *et al.*[11] Fixations were terminated by dumping the "lollipop" contents into either hot or cold ethanol (Table 2). The various trapping methods used are tabulated (Table 2).

Fixations of $C^{14}O_2$ with *W. punctata* were done with suspensions of the plants washed off agar slants with 0.001 *M* KH_2PO_4. Fifteen-milliliter portions of the suspension were placed in 250-ml. Erlenmeyer flasks in which the $C^{14}O_2$ fixations were carried out. Fixations were terminated by adding ethanol at −40° to the reaction mixture. The *Wolffiae*, still in the cold ethanol, were ground with sand. The soluble material was separated from the debris by centrifugation in the cold. Methods for concentration of the solution and isolation of products were the same as those used for the algae.

Chloroplasts were isolated from spinach by the method of Arnon,[12] extracted with ethanol, and chromatograms of the ethanol concentrate were scanned for pteridines.

Results.—All experiments, unless otherwise stated, were done with *E. gracilis*. A yellow fluorescent product was isolated consistently when C^{14} bicarbonate and hydroxylamine were added simultaneously, even if the cells were killed in hot ethanol (Table 2). When the hydroxylamine was added after the carbonate, just before the alcohol killing, the amount of yellow-fluorescent product was variable (Tables 2, 3).

TABLE 3

EFFECT OF NH_2OH ON 10-SEC. PHOTOSYNTHETIC $C^{14}O_2$ FIXATION BY *E. gracilis**

Molarity of NH_2OH in Reaction Mix	Counts $\times 10^6$/Gm Cells
0	2.39
0.01	3.33
0.03	5.47
0.06	4.39
0.1	3.66
0.2	1.07

* Hydroxylamine and C^{14} bicarbonate were injected simultaneously and after 10-second cells were plunged into ethanol at −40° C.

The radioactive yellow-fluorescing material was eluted from the chromatograms with 0.1 *N* NaOH and its absorption spectrum determined (Fig. 1). The spectrum,

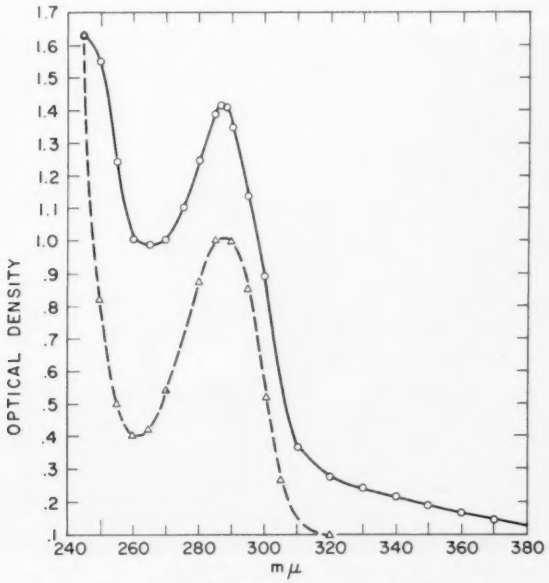

FIG 1.—Ultraviolet absorption spectrum of yellow-fluorescing pteridine in 0.1 *N* NaOH: ○——○ isolated from sonical disrupted *E. gracilis;* Δ— —Δ isolated from hydroxylamine-trapped 10-second $C^{14}O_2$ fixation.

color of fluorescence, and C^{14}-labeling suggest that this material is a reduced conjugated pteridine which contains C^{14}, probably as a one-carbon fragment. When

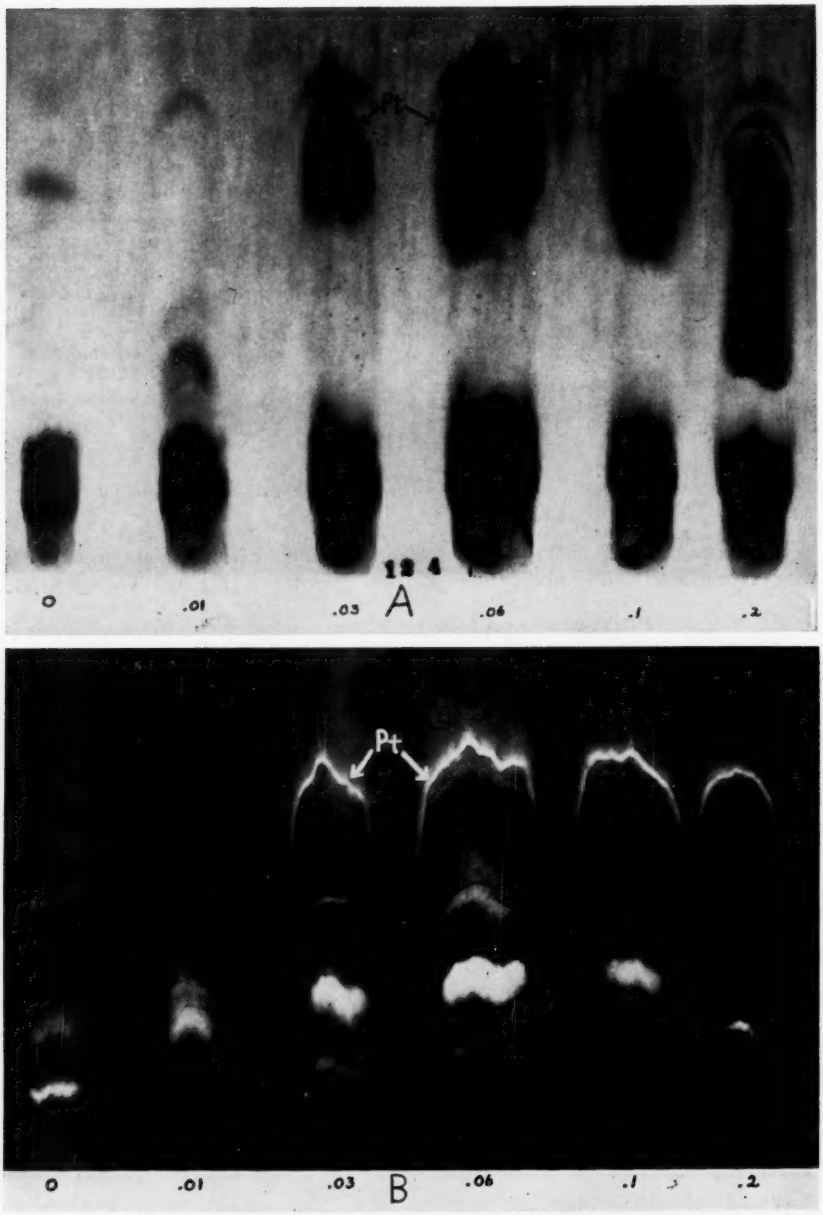

FIG. 2.—One-dimensional chromatogram (developed with butanol: propionic acid) of *E. gracilis* 10-second $C^{14}O_2$ fixations done in the presence of different molar concentrations of hydroxylamine. Numbers along the base of the figures give molar concentrations of hydroxylamine. The C^{14}-labeled pteridine, both on the fluorescence photograph and on the radioautograph, is indicated by *Pt*. *B:* chromatogram viewed with long-wave ultraviolet lamp; *A:* radioautograph of the same chromatogram.

sonic extracts from large quantities of non-radioactive *E. gracilis* were concentrated, a fluorescing material was demonstrated which was similar to the one obtained in the $C^{14}O_2$ fixation experiments when hydroxylamine was used as a trapping agent (Fig. 1). It also is a conjugated pteridine and is present in very small concentrations in the cells.

The amount of hydroxylamine added to the "lollipop" influenced total fixation; at low levels there is an enhancement of fixation, at high levels an inhibition (Table 3). Paired fluorescent photographs and radioautographs of the same chromatogram of the series of 10-second $C^{14}O_2$ fixations are shown in Figure 2.

All organisms used, ranging from a photosynthetic bacterium to a seed plant, yielded a similarly C^{14}-labeled yellow-fluorescing pteridine as a result of short-term fixations (Table 2). In all these organisms a non-fluorescing compound, thus far unidentified, was noted in all two-dimensional chromatograms as a product of hydroxylamine trapping (e.g., Fig. 3, *B*, area marked "unknown").

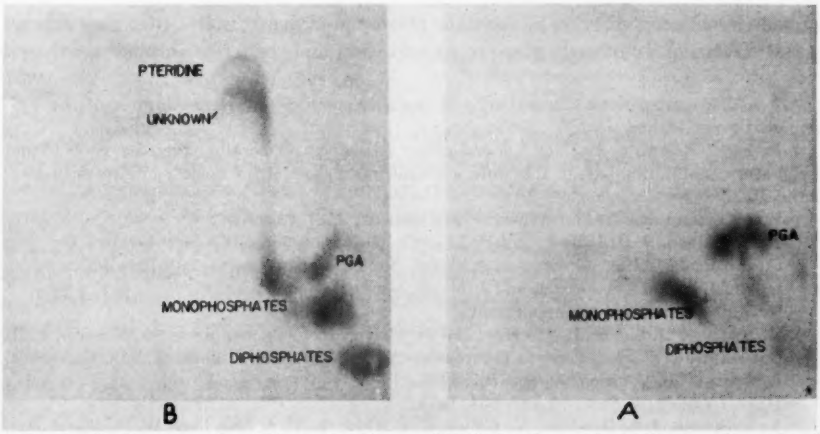

FIG. 3.—Radioautographs of *E. gracilis*, 10-second $C^{14}O_2$ fixation products after two-dimensional chromatography. *A:* no hydroxylamine added; *B:* 0.1 *M* hydroxylamine added at zero fixation time.

In several experiments phenylhydrazine was substituted for hydroxylamine as the trapping agent (Table 2). Once more a radioactive yellow-fluorescing pteridine, which, of course, differed in R_f, was observed on the chromatograms. This compound was readily differentiated from unreacted non-radioactive phenylhydrazine by two-dimensional chromatography.

Small quantities of pteridines were also isolated on chromatograms from alcohol extracts of purified spinach chloroplasts. Though only preliminary studies on their identification have been done, these pteridines appear similar to those isolated from the whole cells of the algae.

Discussion.—To be of basic importance in photosynthesis, which has many common features regardless of whether the "plant" is primitive or advanced, a product should be consistently demonstrable in $C^{14}O_2$ fixations done with diverse groups of organisms. The radioactive yellow-fluorescing pteridine and the non-fluorescing "unknown" (cf. Fig. 3, *B*) are such compounds; they are light-induced fixation

products of all the organisms studied—bacteria, algae, and higher plants. In parallel dark $C^{14}O_2$ fixations in the presence of hydroxylamine, the yellow-fluorescing pteridine consistently appeared but was not radioactive. This, coupled with the demonstration of pteridines in chloroplasts, which have beeen shown to contain all the ingredients necessary for CO_2 fixation,[12] supports the idea that this yellow fluorescent pteridine may function in the early stages of CO_2 fixation. That conjugated pteridines can act as carriers of one-carbon fragments is well known. It is interesting to note that where pteridines do act as one-carbon transferring agents, it is believed that a reduced product of CO_2 is attached to the *N*-10 or the *N*-5 position, as in leucovorin. Gibbs and Kandler[13] have reported that the hexose formed from $C^{14}O_2$ photosynthesis is not symmetrically labeled, as might be required by the direct carboxylation of ribulose-diphosphate by CO_2. These workers have suggested that there is a prior reduction of CO_2 before the addition to pentose. A formyl pteridine could well act as such a transferring compound. The exact nature and function of pteridines in photosynthesis, however, is yet to be determined. One interpretation of the data presented above is that a pteridine acts as a carbon carrier in an early stage of CO_2 fixation and reduction in photosynthesis.

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† Research Associate, Haskins Laboratories. Research collaborator, Brookhaven National Laboratory, and during part of this work a President's Fellow of the Society of American Bacteriologists.

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*THE ENZYMATIC ACTIVITY ASSOCIATED WITH THE PROTEIN IMMUNOLOGICALLY RELATED TO TRYPTOPHAN SYNTHETASE**

By Charles Yanofsky† and Joan Stadler‡

DEPARTMENT OF MICROBIOLOGY, SCHOOL OF MEDICINE, WESTERN RESERVE UNIVERSITY, CLEVELAND, OHIO

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It has been well established in a variety of organisms that gene mutations affect the formation of specific enzymes. In view of the complexity of the gene in terms of numbers of distinct mutational sites,[1] it might be expected that different changes within any one gene would result in different alterations of a specific enzyme or protein. Some enzyme alterations have, in fact, been associated with gene changes,[2–5] and, in one case,[6] a single amino acid substitution has been shown to distinguish the mutant from the normal form of a protein. These instances strengthen the view that the amino acid sequence of each protein may reflect the arrangement of the subunits of its determining gene.

In *Neurospora*, certain mutants defective in the ability to form the enzyme tryptophan synthetase (TSase) are distinguishable from one another on the basis of analyses of the following types: growth in the absence of tryptophan, accumulation of intermediates, reversion frequency, response or lack of response to specific suppressor genes, enzyme activity measurements, and immunological comparisons.[7–11] Since these strains represent mutations of the same gene and are distinguishable from one another, it may be assumed that in some cases the dissimilarities detected result from different alterations of TSase. In this regard the immunological findings are of particular interest because they demonstrate that certain of these mutants form a protein (designated CRM) which may be an inactive or incomplete form of TSase.[10] This protein is immunologically similar to TSase and is absent from at least one mutant which lacks TSase.[10,11] Whether the CRM's formed by distinguishable mutant types differ from one another has not been determined, but differences might be expected if the CRM's represent altered forms of TSase.

Studies with the analogous system in *Escherichia coli* have shown that mutants lacking TSase fall into the same two groups, viz., those which form a cross-reacting protein (CRM) and those which do not.[12] In *E. coli*, mutants in the latter group have two additional characteristics which distinguish them from strains which form CRM; they accumulate indoleglycerol instead of indole,[12, 13] and they lack an enzyme required for the conversion of indoleglycerol phosphate to indole.[14] The absence from these mutants of both CRM and an enzyme involved in the synthesis of indole suggested a second interpretation of the nature of CRM, viz., that it is an enzyme catalyzing an earlier step in tryptophan synthesis.[12] This report is concerned with a study of this possibility.

MATERIALS AND METHODS

The characteristics of the various mutant strains of *E. coli* employed in this

study are summarized in Table 1, in which the probable pathway of tryptophan synthesis in this organism is also indicated. With the exception of the stocks designated "B" (derived from the B strain of *E. coli*), the mutants listed were obtained from the K-12 strain of *E. coli*. Mutant T-3 was considered equivalent to the wild type for the purposes of this study. It forms large amounts of TSase and the other enzymes involved in tryptophan synthesis when grown on low levels of anthranilic acid.[14]

TABLE 1

CHARACTERISTICS OF THE STRAINS EMPLOYED

STRAIN	GROWTH ON*				ACCUMULATION	ENZYME ACTIVITY ABSENT†
	0	0 + A	0 + In	0 + T		
Wild-type K-12	+	+	+	+	...	...
T-3	−	+	+	+	?	?
T-4, T-8	−	+	+	+	InG	A
T-41, T-84	−	−	−	+	InG	B, TSase
$td_{2,3,4,5}$, B-82	−	−	−	+	In	TSase
B/IT	−	−	−	+	?	A, B, TSase‡
B/IT-N	−	−	−	+	InG	A, B, TSase

⟶ anthranilic acid ⟶ indoleglycerol phosphate ⟶ indole ⟶ tryptophan

* 0 = unsupplemented minimal medium; A = anthranilic acid; In = indole; T = tryptophan; InG = indoleglycerol.
† A and B are enzyme components required for the conversion of indoleglycerol phosphate to indole.
‡ Also lacks the enzymes involved in the conversion of anthranilic acid to indoleglycerol phosphate.

Tryptophan Synthetase Assay.—TSase activity (the coupling of indole and serine to form tryptophan) was determined as described previously,[15] with the following minor changes: (1) tris buffer, pH 7.8, was substituted for phosphate buffer, and (2) 0.03 ml. of a saturated solution of sodium chloride was added per milliliter of incubation mixture. Under these conditions the activity of the TSase of *E. coli* is approximately double that which is observed in the presence of phosphate buffer alone. Immunological tests indicate that this increase is not accompanied by an increase in TSase antigen. One unit of TSase activity is defined as the amount of enzyme which will convert 0.1 μmole of indole to tryptophan in 20 minutes at 37° C. in an incubation mixture containing tris buffer, sodium chloride, pyridoxal phosphate, indole, and serine. Specific activity is expressed as units of TSase per milligram protein. Protein was determined by the method of Lowry *et al.*[16]

Component B Assay.—As will be shown subsequently, two enzyme components are required for the conversion of indoleglycerol phosphate to indole. One of these has been arbitrarily designated component A, and the second, component B. Either activity can be determined in the presence of an excess of the other. The incubation mixture employed in assaying component B activity contained 0.15 μmoles of indoleglycerol phosphate; 0.1 ml. of 0.5 *M* phosphate buffer at pH 7.0; 0.05 ml. of 5×10^{-2} *M* NH_2OH; and a suitable excess of partially purified component A in a final volume of 0.5 ml. Reaction mixtures were incubated for 20 minutes at 37°, 0.1 ml. of 1 *N* sodium hydroxide was then added to stop the reaction, and the indole formed was subsequently extracted with toluene and determined colorimetrically.[15] One unit of component B activity is defined as the amount of enzyme which will form 0.1 μmole of indole from indoleglycerol phosphate in 20 minutes at 37° under the foregoing conditions. Specific activity is expressed as units of B per milligram protein.

Preparation of Antisera.—Antibodies were produced in rabbits in response to the injection of partially purified preparations of TSase or CRM.[12] The antisera were heated for 30 minutes at 56°, fractionated with ammonium sulfate, and then dialyzed before use. In some cases, antisera were repeatedly treated with portions of extracts of K-12 mutants (T-84 and T-41) lacking both CRM and TSase to remove antibodies to other *coli* antigens. The absorbed antisera were then heated, fractionated with ammonium sulfate, and dialyzed before use.

Neutralization of TSase.—Antibodies to TSase or to CRM are capable of completely neutralizing TSase activity.[12] Over a considerable portion of the neutralization curve (see Fig. 1), however, some of the enzyme is precipitated without being neutralized (from 0.08 to 0.2 ml. antiserum). It is also evident from Figure 1 that the amount of enzyme activity remaining in the supernatant solution decreases linearly as increasing amounts of antiserum are added. As previously reported,[12] 15 minutes is sufficient for an antiserum to exert its maximal inhibitory effect.

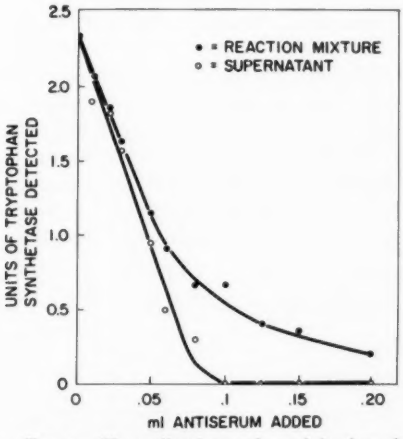


FIG. 1.—Neutralization and precipitation of TSase. Mixtures of TSase and antiserum (a 1–20 dilution) were incubated for 18 hours at 4° C. Aliquots of the reaction mixture and the supernatant solution obtained after centrifugation were then assayed for TSase activity.

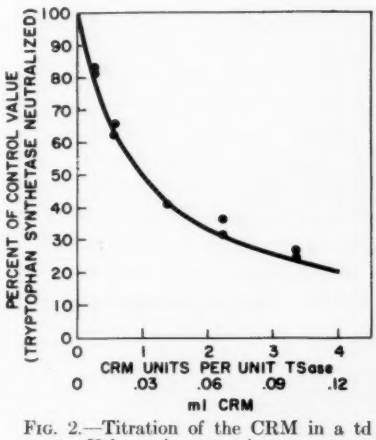


FIG. 2.—Titration of the CRM in a td extract. Values given are in percentages of the amount of TSase neutralized in control tubes. The solid line is the theoretical curve, and the points plotted were obtained experimentally with a 1–9 dilution of an extract of strain td_4.

Neutralization of Component B.—Antisera to TSase or to CRM, whether absorbed or not, are capable of precipitating component B. However, only a fraction of the B activity which is precipitated by antibody is neutralized.[17] In determinations of antibody–component B interactions, therefore, precipitates were removed by centrifugation (following 40-hour incubations), and only supernatant solutions were assayed for component B activity.

Immunological Assay of CRM.—Two methods were used for estimating the amount of CRM in various mutant extracts and preparations. In the first method, unknown amounts of CRM were preincubated with an excess of antibody, and, after a suitable period, the amount of antibody remaining unneutralized was determined.[12] In the second or "competition" method, aliquots of CRM solutions

were thoroughly mixed with a known amount of TSase prior to the addition of antibody. Antibody was then added, and the reduction in neutralization of TSase activity resulting from the presence of CRM was determined. In this assay procedure, which gave more reproducible results than the first method, one unit of CRM was defined as the amount of material which was antigenically equivalent to (i.e., would combine with as much antibody as) one unit of TSase. The theoretical curve based on this assumption is shown in Figure 2, as well as the experimental points obtained in a typical titration of the CRM in an extract of mutant td_4. Assays by the second method were reproducible to within 10–20 per cent, and this procedure was accordingly used in instances where a quantitative titration of CRM was desired. CRM specific activity is expressed in units of CRM per milligram protein.

RESULTS

In initial studies on formation of indole from indoleglycerol phosphate by mutant extracts it became apparent that at least two enzymes are required for this conversion. This is illustrated by the data in Table 2. Extracts of neither strain T-84 nor strain T-8 alone were capable of converting indoleglycerol phosphate to indole. A mixture of these two extracts, however, rapidly carried out this conversion. We have arbitrarily designated the active T-84 and T-8 components as A and B, respectively. It can also be seen from Table 2 that extracts of wild-type strains contain two distinct components, both of which are essential for the conversion of indoleglycerol phosphate to indole. One of these components, A (determined by mixing experiments with T-84 and T-8 extracts), remains in the supernatant solution when extracts are treated with acetic acid until precipitation just occurs. The second component, B, is more stable than A to heating at 60°.

TABLE 2

SEPARABILITY OF COMPONENTS A AND B

	Extract	μG Indole Formed*
1.	T-84 alone (A)	0
2.	T-8 alone (B)	0
3.	1 plus 2	12.1
4.	Acid supernatant (wild type)	0
5.	Heat supernatant (wild type)	1.2
6.	4 plus 5	8.8

* Under standard conditions employed in assaying component B.

The present study is concerned with the possibility that CRM is a participant in the conversion of indoleglycerol phosphate to indole. If CRM is involved in this conversion, *it must be component B and not component A, since some mutants which lack CRM—T-41* and T-84—*form component A*.

The B and CRM Content of Mutant Extracts.—To study the relationship between B and CRM, extracts of five mutants containing CRM but lacking TSase were assayed for component B and CRM levels. The results of these analyses are presented in Table 3. It can be seen that, with the exception of strain td_3, the B/CRM ratios observed were fairly constant. Approximately the same ratio was found in extracts of cells grown in the presence of high levels of tryptophan;

under these conditions the formation of both component B and CRM is inhibited. Strain td_3 consistently exhibits somewhat higher B/CRM ratios than the other td mutants. This observation and other data which will be presented elsewhere indicate that in extracts of mutant td_3 there is slightly more component B activity per unit of CRM than in other mutant extracts.

TABLE 3

B AND CRM CONTENT OF VARIOUS MUTANT EXTRACTS

EXTRACT	CRM SPECIFIC ACTIVITY	B SPECIFIC ACTIVITY	B/CRM × 100
td_2	71.5	1.7	2.4
td_2	87.5	2.15	2.5
td_2 (high tryp.)*	1.6	0.04	2.5
td_3	72	2.6	3.6
td_3	41.3	1.35	3.3
td_4	14	0.33	2.3
td	42	0.93	2.2
td_5	14	0.34	2.4
B-82	82	2.3	2.8

* Grown on a medium containing 200 μg L-tryptophan/ml.

Fractionation and Comparison of the Stability of B and CRM.—As a further test of the association of component B activity with CRM, fractionation experiments were performed. The results of a typical fractionation are summarized in Table 4. Here and in several similar experiments, all the fractions obtained (representing about an 80-fold spread in B-specific activity) showed approximately the same B/CRM ratio, suggesting that these two activities were inseparable under the various conditions employed.

TABLE 4

FRACTIONATION OF COMPONENT B AND CRM*

FRACTION	CRM/ML	B/ML	B SPECIFIC ACTIVITY	B/CRM ×100
Starting material	728	21	0.53	2.9
25% amm. sulfate	1,534	45	1.6	2.9
45% amm. sulfate	300	9	0.23	3
25%, 53°, 2 min.	1,391	43.5	2.2	3.1
20% amm. sulfate	2,200	75	10	3.4
40% amm. sulfate	770	22.5	2.4	2.9
20% calcium phosphate gel supernatant	1,100	34.5	16	3.1

* The starting material was treated with protamine sulfate, and the supernatant solution obtained after centrifugation was adjusted to pH 6.0. Solid ammonium sulfate was added to 25 per cent of saturation and the precipitate which formed was collected by centrifugation. The supernatant solution was fractionated further at 45 per cent of saturation. The 25 per cent fraction was heated at 53° for 2 minutes, and the precipitate was discarded. The supernatant solution was fractionated with ammonium sulfate at 20 per cent and 40 per cent of saturation. The 20 per cent fraction was purified further by calcium phosphate gel treatment.

Sensitivity of B and CRM to heat and alkaline pH was tested and both B and CRM were found to be affected to the same extent (see Table 5). Similarly, determinations on the supernatant solutions obtained following precipitation of extracts with dilute acetic acid indicate that, although only small amounts of B activity remain, corresponding levels of CRM are present. Partially purified preparations of component A (from an extract containing both components) have only *trace* amounts of component B but contain a *corresponding* amount of CRM. Thus these tests,too, suggest an association of B activity with CRM.

TABLE 5

INACTIVATION OF B AND CRM

TREATMENT	PER CENT DECREASE IN B	PER CENT DECREASE IN CRM
Alkali at pH 11 for 5 min.	88	89
Heat at 60° for 10 min.	53	60
Heat at 52° for 17 min.	72	68
Acid supernatant	90	87

Neutralization of the TSase in Extracts with High Levels of B.—If component B is actually CRM, it would be expected that the TSase in extracts of mutants which have a B/TSase ratio higher than that observed in extracts of wild type strains would be relatively more difficult to neutralize with antiserum. That is, if, in addition to TSase, a mutant extract contains an excess of CRM, a given amount of antiserum should be less effective in inhibiting its TSase than in inhibiting the TSase in a wild-type extract. The results presented in Table 6 show that mutants which lack component A (T-4 and T-8) do exhibit a B/TSase ratio which is higher than that observed in wild-type extracts. The fifth column of this table shows that a given amount of antiserum is much less effective in inhibiting the TSase of T-4 and T-8 extracts than in inhibiting the TSase activity of the wild-type preparation. Using the B/CRM ratio characteristic of mutants td_2, td_4, and td_5 and the B/TSase ratio characteristic of wild-type strains, it is possible to calculate the reduction in neutralization expected with T-4 and T-8 extracts (assuming that the excess B is equivalent to a corresponding amount of CRM). Comparison of the values in the last two columns indicates that the experimental values compare favorably with those calculated on this basis.

TABLE 6

NEUTRALIZATION OF TSASE OF STRAINS LACKING COMPONENT A

EXTRACT	SPECIFIC ACTIVITY TSase	SPECIFIC ACTIVITY B	B/TSASE × 100	TSASE UNITS PER CENT OF CONTROL	NEUTRALIZED PER CENT EXPECTED*
Wild type					
K-12 (control)	6.3	0.17	2.7†	100	
T-4	1.1	0.12	10.9	26	24
T-8	3.5	0.5	14.3	15	18

* Based on theoretical curve in Fig. 2.

† It may be noted that this ratio is exactly the same as the B/CRM ratios obtained with extracts of mutants td_2, td_4, and td_5. This finding indicates that, per unit of antigenic material, there is as much B activity in mutants as in the wild-type strain.

In a further study, neutralization of the TSase in a T-4 extract was compared with neutralization of a mixture containing T-3 TSase and td_2 B in amounts which reproduced exactly the B/TSase ratio of the T4 extract. The results of this experiment (Fig. 3) show that the T-4 TSase and the TSase in the T-3-td_2 mixture were both inhibited to the same extent by the antiserum employed.

Comparison of the Neutralization of B and CRM.—In other experiments, the precipitation of component B and CRM by absorbed antisera was compared. The results of two such experiments are presented in Figure 4. Both CRM and component B were affected to approximately the same extent by increasing amounts of antiserum, and both were completely removed at the same point. In similar tests, antiserum to td_2-CRM (absorbed) also precipitated both CRM and component B to the same extent. When excesses of antibody were incubated (2

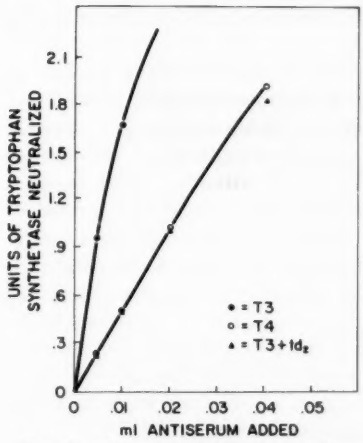


FIG. 3.—Comparison of the neutralization of the TSase in a T-4 extract and in a mixture containing T-3 TSase and td_2 B. The mixture contained T-3 TSase and td_2 B in amounts which reproduced exactly the B/TSase ratio of the T-4 extract. The neutralization curve obtained with T-3 TSase alone is included for comparison.

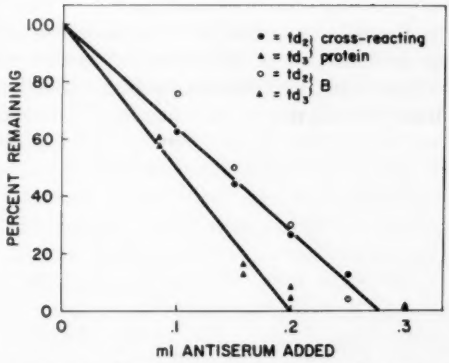


FIG. 4.—Comparison of the neutralization of component B and CRM in td extracts. Varying amounts of absorbed TSase-antiserum were incubated with constant amounts of td_2 or td_3 extracts in the presence of glutathione and pyridoxal phosphate. After 2 days at 4° C. the reaction mixtures were centrifuged and the supernatant solutions assayed for remaining activity.

days at 4°) with component B and CRM, the antibody remaining in the supernatant solution following centrifugation also precipitated component B and CRM to approximately the same extent. Although both CRM and component B were generally precipitated to the same degree by the various antisera employed, a significant difference was often observed in the region of high antigen excess. In this region, somewhat more B activity was detected in the supernatant solution than was expected on the basis of the amount of CRM which was removed. This may be due to the often observed partial solubilization of an antigen-antibody precipitate by excess antigen.

DISCUSSION

Biochemical studies with *E. coli*[14, 18] have implicated the following reactions as steps in the biosynthesis of tryptophan:

$$\text{Indole-3-glycerol phosphate} \xrightarrow[\text{component A}\ +\ \text{component B}]{} \text{indole} \xrightarrow[\text{TSase}]{+\ \text{serine}} \text{tryptophan}$$

The data presented in this paper demonstrate a striking similarity in the behavior of component B, an enzyme required for the conversion of indoleglycerol phosphate to indole, and CRM, a protein immunologically related to TSase. On the basis of the similarities observed, it appears likely that component B activity and CRM represent, respectively, the enzymatic and immunological properties of the same protein.

In evaluating this conclusion in terms of the relationship between CRM (B) and TSase, two possibilities are being considered: (*a*) that CRM (B) and TSase are distinct but closely related proteins and (*b*) that one protein normally exhibits

both activities (B and TSase) and that CRM is a mutationally altered form of this protein that retains only component B activity.

The different types of mutants which have been obtained (lacking component B and/or TSase) are of importance in considering the foregoing possibilities. These classes and the class lacking component A are indicated in Table 7, together with the mutants present in each. Although the mutants listed were obtained in several laboratories and from two wild-type strains of *E. coli*, one class of mutants is noticeably absent, the class lacking component B alone. This class is replaced by a category containing strains in which both component B and TSase are absent. All nine of the mutants in this class have been examined in reversion experiments, and all do revert. However, when they do, both activities are invariably regained at the same time. It would appear, therefore, that these strains represent instances in which two enzymatic activities are lost and regained as a result of single mutational events. The fourth category in Table 7 includes mutants in which portions of the genic region controlling tryptophan biosynthesis have been lost by deletions; their behavior is not relevant to present considerations.

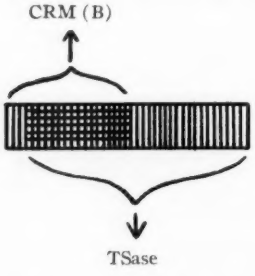
TABLE 7

GROUPING OF MUTANTS LACKING COMPONENTS A, B, OR TSASE

LACK A	LACK B AND TSASE	LACK TSASE	LACK A, B, AND TSASE
T-4, T-8	T-41, T-84	B-82*	B/IT
T-26, T-106	WRT-1, WRT-2	td_2, td_3	B/ITN
B-42,* B-48*	B-81,* B-84*	td_4, td_5	..
B-4*	B-9,† B-10,† B-11†	...	...

* Obtained from Dr. J. Gots.
† Obtained from Dr. P. D. Skaar.

Since one class of mutants (excluding the deletion mutants) lacks both component B and TSase, it appears unlikely that CRM (B) and TSase are different proteins under the control of separate genes. They could, however, be two distinct proteins formed by a complex of two neighboring genes with an overlapping functional region[19] (see diagram). Mutations within the overlap region could affect the formation of both CRM (B) and TSase, thus explaining the origin of one class of mutants, while mutations within the B or TSase-specific regions could affect these activities separately. If the B-specific region were very small in comparison with the rest of the gene complex, mutants lacking only component B would be expected to be very rare. This model has the advantage that it would also explain the tendency of the various genes involved in tryptophan synthesis in *Salmonella typhimurium* and *E. coli* to remain clustered.



The available data do not exclude the possibility, however, that both component B activity and TSase activity are associated with the same protein and that the formation of this protein is controlled by a single gene. Mutations within this gene could presumably affect it so that the ability to form the protein bearing both activities is lost or, when less drastic, could affect only the portion of the gene concerned with the TSase reaction. To

explain the absence of mutants lacking component B alone, it may be assumed that mutations in the genic region which affects B activity result in protein alterations which eliminate both the immunological identity and the TSase activity of the protein. Consistent with this interpretation is the possibility suggested by recent studies with *Neurospora*[20] that the formation of tryptophan from indoleglycerol phosphate (or a compound derived from indoleglycerol phosphate) occurs on one enzyme surface, with enzyme-bound indole generated from indoleglycerol phosphate serving as the true intermediate rather than free indole.

Studies designed to distinguish between the alternative possibilities are now in progress.

SUMMARY

Enzymatic and immunological studies with mutants of *E. coli* have provided evidence suggesting that the cross-reacting protein related to tryptophan synthetase can catalyze a reaction involved in the conversion of indoleglycerol phosphate to indole. The possibility that this reaction and the coupling of indole and serine are normally catalyzed by the same enzyme, tryptophan synthetase, is discussed.

The authors are indebted to Miss Thelma Anderson for her technical assistance and to Doctors H. Gest, A. Stavitsky, and J. Spizizen for many helpful suggestions.

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† Present address: Department of Biological Sciences, Stanford University, Stanford, California.

‡ U.S. Public Health Service Research Fellow of the National Cancer Institute.

[1] See G. Pontecorvo in *Cold Spring Harbor Symposia Quant. Biol.*, Vol. **21**, 1956, for a discussion of the data bearing on this point.

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A PRELIMINARY INVESTIGATION OF PSEUDOALLELISM AT THE NOTCH LOCUS OF DROSOPHILA MELANOGASTER

By W. J. Welshons

BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY,* OAK RIDGE, TENNESSEE

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In order to place the purpose and results of this investigation in their proper perspective, it will be necessary to present a short summary of the existing information concerning the *Notch* locus. In *Drosophila melanogaster* this locus has yielded both dominant and recessive mutants. The dominant class of *Notch* mutants will be discussed first. Then some pertinent information concerning four recessive mutants and their interaction with the dominant *Notches* will be presented.

Notch is a sex-linked dominant which is lethal in hemizygous males. Heterozygous females usually display notched wings, thickened wing veins, and certain bristle abnormalities. Many *Notch* mutations are associated with deficiencies[1, 2], translocations, or inversions in which the cytological break points are in the vicinity of salivary chromosome band 3C7; some of these mutants are variegated-type *Notches*.[3] Other *Notch* mutations are deficiencies for various lengths of the salivary chromosome. All but a few of these, which may be stable-type position-effect *Notches*, lack at least the 3C7 band, and some are deficient for only 3C7.[3, 4] The correspondence between the loss of band 3C7 and the production of a *Notch* phenotype has led to the conclusion that the locus of *Notch* is probably contained within this single band.[3] Although an appreciable number of *Notch* mutants exist which do not possess a visible deletion, it is possible that these mutants are subvisible deficiencies for a portion of band 3C7.

In addition to the variety of *Notch* mutants, there exists at approximately the same genetic and cytological location a series of recessive mutants called *facet* (*fa*), *facet-notch* (fa^{n}), *facet-notchoid* (fa^{no}), and *split* (*spl*). Both *facet* and *split* affect the eyes of *Drosophila*, but the two phenotypes differ in detail and can be separated with ease.[5, 6] *Facet-notch* and *facet-notchoid* are wing mutants, but again the phenotypes are separable.[5, 6] The heterozygous combinations between *fa*, fa^{n}, and *spl* have been reported to result in a wild phenotype;[5, 6] one would presume that the mutants are non-allelic. However, an interpretation of allelism is favored by the fact that recombination between these mutants had not been reported. Consistent with this interpretation is some evidence obtained by Glass[5] for an allelic relationship between *fa* and fa^{n}, and the results obtained by Bauer,[6] which suggest an allelic relationship between fa^{n} and fa^{no}. In fact the detailed experiments of the latter investigator indicated at least a slight degree of interaction between all four of the mutants *fa*, fa^{n}, fa^{no}, and *spl*. Bauer went so far as to postulate the presence of a gene doublet which had arisen in the course of evolution in the form of a tandem duplication involving a part of salivary band 3C7. He suggested that the mutants *fa*, fa^{n}, and fa^{no} were associated with one locus and the *spl* mutant with the other. It should be noted that, in order to explain his results, Bauer had invoked a pseudoallelic interpretation, although the term "pseudoallele" was not used because it had not yet acquired its present connotation.

The information obtained from heterozygous combinations of *Notches* with recessive mutants is as follows: The N/fa^{no} heterozygotes are lethal, while homozygous *facet-notchoid* females and hemizygous males are viable and fertile.[6] When *fa*, fa^{n}, and *spl* are combined with a *Notch*, the resulting heterozygous females are *Notch* in phenotype, and, in addition, there is a pseudo-dominant expression of the recessive mutant.[5, 7] The phenotypic expression of a recessive mutant in the heterozygous condition has often been interpreted to mean that the normal wild-type locus has been deleted from the other chromosome. If a *Notch* mutation represents the deletion of part or all of band 3C7, then it is possible that *fa*, fa^{n}, fa^{no}, and *spl* all lie within this salivary band.[4, 6]

In summation we can say that all the dominant and recessive mutants at this locus appear to be localized cytologically in salivary band 3C7. All the heterozygous combinations of the recessive mutants show at least a slight degree of interaction, and these mutants might be considered allelic. A mutation to *Notch* might result from the deletion of this locus. On the other hand, Bauer's interpretation is essentially a pseudoallelic one, and, on this basis, recombination between the mutants is expected to occur, although none had been reported.

In the experiments reported here an attempt was made first to detect recombination between three of the recessives (the mutant *facet-notch* has not been used because it is presently carried on a chromosome which also bears *In[1]delta-49*). The results of these experiments led to further investigations, which are yielding unexpected information on the behavior of this complex locus.

TABLE 1

THE EVIDENCE FOR RECOMBINATION BETWEEN *fa*, fa^{no}, AND *spl*

CROSS	♂ RECOMBINANTS	APPROXIMATE TOTAL ♂♂	RECOMBINATION (PER CENT)
$\frac{y\ w\ \ +\ spl\ sn}{+\ +\ fa^{no}\ +\ +} \times fa^{no}$	{4 *y w* 2 fa^{no} *spl sn*}	9,400	0.06
$\frac{y\ w\ +\ fa^{no}\ sn}{+\ +\ fa\ +\ +} \times fa$	{2 *y w* 1 *fa* fa^{no} *sn*}	8,100	0.04
$\frac{y\ w\ +\ spl\ sn}{+\ +\ fa\ +\ +} \times y\ w\ spl\ sn$	{4 *y w* 1 *fa spl sn**}	6,100	0.1†

* Phenotypically *spl sn*.
† Calculated on the assumption that the *fa spl* combination could not be recognized.

As shown in Table 1, crossing over occurred between the mutants fa^{no} and *spl* in the cross *y w spl sn* / $fa^{no} \times fa^{no}$. The *y w* ♂♂ and fa^{no} *spl sn* ♂♂ are to be expected from such a cross if the mutant fa^{no} is located to the left of the mutant *spl*. In the next cross, *y w* fa^{no} *sn* / *fa* × *fa*, the production of *y w* ♂♂ and *fa* fa^{no} *sn* ♂♂ indicates that the mutant *fa* is localized to the left of the mutant fa^{no}. Hence *fa* must be to the left of *spl;* and in the cross *y w spl sn* / *fa* × *y w spl sn*, one would expect to find males that are *y w* and *fa spl sn*. As indicated, the *y w* ♂♂ were recovered. It was surmised that the *facet split singed* phenotype might not be recognized because the extreme rough-eye characteristic of *split* might conceal the milder rough-eye expression of *facet*. However, a few *split-like* males with *singed* bristles were observed which had a small nick in one wing. Since *facet* flies, showing their characteristic mutant eye type, occasionally have small nicks or notches in their wings, it seemed likely that some of these

split-like males might also carry *facet*, although the nicks in the wings might be due to other factors. Accordingly, they were crossed to homozygous *facet* females, and one of them did give *facet* daughters. This male was presumably the *fa spl sn* type. If this recombinant chromosome carries both mutants, it should be possible to separate them again.

For this purpose the cross $w^a + + rb + / + (fa?)\ spl + sn$ ♀♀ × $w^a + spl\ rb +$ ♂♂ was made. If *facet* is present, it should be possible to obtain *facet ruby* males. The cross yielded eleven of these expected recombinants out of an estimated 28,300 males (approximately 0.08 per cent recombination).

Since crossing over occurs between these three mutants, they are clearly not allelic. The slight interaction between the recessives and the pseudo-dominance observed even when combined with *Notch* mutants which bear no detectable cytological deficiency, suggests that these loci constitute a new pseudoallelic series similar to the *Star-asteroid* and *bithorax* series reported by Lewis.[8]

It is interesting to note that in the case of the *lozenge* pseudoallelic array, coupled pseudoalleles are clearly more extreme in phenotype than the single mutants, even when one of the two alleles is often indistinguishable from wild type.[9] There might be a slight tendency toward this type of reaction in the case of the *fa*, fa^{no}, *spl* series. Homozygous *fa* fa^{no} females and hemizygous males have typical *facet-notchoid-like* wings. However, the eyes are slightly rougher than those of *facet* males and females. The double mutant *fa spl* is very similar to *split*. The fa^{no} *spl* combination results in *facet-notchoid-like* wings, but the rough eyes are slightly reduced in size when compared to the single mutant *split*. Although a tendency toward a more extreme expression is to be observed in the combinations *fa* fa^{no} and fa^{no} *spl*, in no case is the double mutant decidedly more extreme than one would expect by combining the two separate phenotypes.

An attempt was made to detect crossing over between one of the recessives and a dominant *Notch*. Recombination might be possible if one chose a *Notch* which was not cytologically deficient for salivary band 3C7. Accordingly, six *Notches* which do not possess a visible cytological deficiency were chosen for further investigation. Five of these were of X-ray origin and belong to the *264* series of Demerec.[10] The remaining one was spontaneous in origin and is designated as N^{Co}.[11]

Females heterozygous for *spl* and *N* were mated to *spl* males. In the F_1 of such a cross, *split* flies of both sexes and *Notch-split* females are expected. As shown in Table 2, experiment 1, all but one of the different heterozygotes produced at least one expected exception that was neither *Notch* nor *split* out of a total of approximately 25,000 offspring from each heterozygous combination. The presence of the mutant *yellow* in two of the crosses indicated that if the exceptions arose by crossing over between *Notch* and *split*, the mutant *split* must be situated to the left of the dominant *Notch*.

To test this possibility, females were produced which carried *N* or *y N* (recombination value, 3 per cent) on one chromosome and the mutants *spl* and *rb* (recombination value, 4 per cent) on the other. They were crossed to *spl rb* males. If the assumption concerning crossing over between *split* and *Notch* is correct, the exceptional offspring from *N* + / *spl rb* ♀♀ should carry the mutant *ruby*, and those from *y N* + / + *spl rb* ♀♀ should carry both *yellow* and *ruby*.

TABLE 2

SUMMARY OF EXCEPTIONAL OFFSPRING OBTAINED FROM FEMALES HETEROZYGOUS FOR *N* AND *spl**

EXPERIMENT I		EXPERIMENT II	
Cross	Exceptional Chromosomes	Cross	Exceptional Chromosomes
$\frac{N^{Co}}{spl} \times spl$	2 +	$\frac{N^{Co}+}{spl\ rb} \times spl\ rb$	5 + *rb*
$\frac{N^{264-40}}{spl} \times spl$	4 +	$\frac{N^{264-40}+}{spl\ rb} \times spl\ rb$	4 + + 1 + *rb*
$\frac{y\ N^{264-47}}{+\ spl} \times spl$	1 y +	$\frac{y\ N^{264-47}+}{+\ spl\ rb} \times spl\ rb$	2 *y* + +
$\frac{y\ N^{264-103}}{+\ spl} \times spl$	1 y +	$\frac{y\ N^{264-103}+}{+\ spl\ rb} \times spl\ rb$	1 + + *rb* 1 ? + *rb*†
$\frac{y\ N^{264-107}}{+\ spl} \times spl$	0	$\frac{y\ N^{244-107}+}{+\ spl\ rb} \times spl\ rb$	1 *y* + +
$\frac{N^{264-109}}{spl} \times spl$	1 +	$\frac{N^{264-109}+}{spl\ rb} \times spl\ rb$	0

* Approximately 25,000 offspring were examined from each *Notch* and *split* combination.
† Lost before the presence or absence of *y* could be determined.

The results are summarized in Table 2, experiment 2. The exceptional offspring obtained from *N* + / *spl rb* ♀♀ were unexpectedly of two types; some carried the mutant *ruby*, and some did not. The exceptions obtained from *y N* + / + *spl rb* ♀♀ carried either *yellow* or *ruby;* the expected exceptional type carrying both markers did not appear.

Contrary to the original expectations, these crosses demonstrate that exceptional offspring, which are neither *Notch* nor *split*, can be obtained from heterozygous females without recombination between the closely linked markers on either side of the region in question. These results do not eliminate the possibility that recombinational exceptions may also arise. In fact, more intensive experiments, which will be reported elsewhere, have definitely indicated that recombination is frequently associated with the appearance of an exception. In this respect the data resemble the results reported for *Neurospora* by Mitchell[12] and St. Lawrence.[13]

The production of recombinant wild-type exceptions from *N* / *spl* heterozygotes suggests that the *Notch* chromosome is not deficient for the *spl* locus. And the recovery of non-recombinant spl^+ and N^+ chromosomes suggests that neither mutant is of the nature of a deficiency. These observations, plus the knowledge of the existence of variegated-type position-effect *Notches* which are non-deficient, suggest that a *Notch* mutation need not be associated with a deletion of the loci *fa*, fa^{no}, and *spl*. Therefore, these preliminary experiments support the conclusion of Demerec[3] that *Notch* mutations may result from a suppression of genetic activity as well as by a deletion of genetic material. Furthermore, this genetic suppression must extend over a linear series of at least three loci and not over just a single locus, as the previous observations seemed to indicate.[3]

The *Notch* mutants used in these experiments are probably inactivations of a series of at least three loci known to be arranged in a linear order. Therefore, it becomes of interest to determine the cytological position of the three loci on the salivary map. In this connection the heterozygotes of $Df(1)rst^2$ with each of the recessives *fa*, fa^{no}, and *spl*, and with $Df(1)N^{264-39}$ are revealing. Schultz[10] has reported that $Df(1)rst^2$ is deficient for bands 3C4 – 3C6, and $Df(1)N^{264-39}$ is re-

ported to be deficient for only 3C7.[4] When $Df(1)rst^2$ was combined with each of the recessives, no pseudo-dominant effect was observed. The N^{264-39}/rst^2 combination was viable and *Notch* in phenotype. It would appear that the three recessive loci are not contained within salivary bands 3C4 – 3C6. One might expect them to be contained within one or more bands to the right of 3C6.

The two deficiencies were rechecked cytologically. $Df(1)N^{246-39}$ certainly appears to be deficient for only 3C7. Unfortunately, the cytology of $Df(1)rst^2$ could not be accurately determined. The region in question appears to be deficient, but the extent of the deficiency could not be defined. Therefore, the loci of *fa*, fa^{no}, and *spl* are tentatively assigned to one or more bands to the right of 3C6.

Summary.—Recombination has been obtained between the mutants *fa*, fa^{no}, and *spl*, all of which are known to be localized cytologically in a short region of the X-chromosome. Since these recessives show some interaction with each other and have a pseudo-dominant expression in combination with *Notches* which are not visibly deficient, it appears that this complex in the *Notch* region represents a new case of pseudoallelism.

Six different *N* / *spl* heterozygotes produced exceptional wild-type progeny. Some of these exceptional offspring are not associated with recombination. The production of exceptional wild types from heterozygotes of *N* and *spl* supports the conclusion that *Notch* mutations can occur without the deletion of genetic material. Even in such cases, however, the effect of the *Notch* mutation extends over a linear series of loci *fa*, fa^{no}, and *spl*.

The three recessive loci are apparently distributed over a series of bands to the right of 3C6, or they are all contained within band 3C7.

* Operated by Union Carbide Nuclear Company for the United States Atomic Energy Commission.

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PRINCIPLE OF MINIMUM BENDING OF LOCALIZED AND DELOCALIZED ORBITALS: ETHANE BARRIER AND RELATED EFFECTS

By Henry Eyring, George H. Stewart, and Richard P. Smith

DEPARTMENT OF CHEMISTRY, UNIVERSITY OF UTAH, SALT LAKE CITY, UTAH

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It appears to us that the main principles of chemical bonding may be formulated, in order of importance, as follows: (1) an electron seeks lowered potential energy by moving close to a nucleus; (2) an electron seeks lowered kinetic energy by lengthening its path by moving over several nuclei; (3) an electron seeks lowered kinetics energy by avoiding orbital bending. Governing the operation of these effects is, of course, the exclusion principle.

Effect 1, of the order of hundreds of kcal/mole, stabilizes inner-shell electrons close to nuclei in a molecule. Also, it is responsible for the small stabilization due to the partial ionic character of a bond. Effect 2, of the order of chemical bonding energies, is largely responsible for the formation of covalent bonds, as in H_2, and for further stabilizations due to delocalization effects, as in benzene. It is easily shown that long path leads to lowered kinetic energy, for example, by considering the well-known problem of a particle in a box, a model which has found much use in the one-dimensional, free-electron description[1] of conjugated systems, and even in rough descriptions of the simple electron-pair bond. This one-dimensional model does not take effect 3 into account, so that only the length of a path, and not its shape is supposed to determine the energy. We propose that effect 3 is of fundamental importance in the consideration of the fine structure of molecules and that it will largely account for various effects of the order of a few kcal/mole, including the as yet incompletely explained[2] rotational barrier in ethane.

To understand effect 3, we examine the kinetic energy part of the Hamiltonian operator. In the free-electron method, only one of the second derivatives is retained. It corresponds to curvature in one dimension and is made small by having a long path. But a more careful treatment must include the other two derivatives, which are smallest for minimum orbital bending. The energy of bending of any triatomic molecule is a measure of effect 3.

In view of effect 2, the electrons in ethane will delocalize, to a small extent, over the four-atom paths H—C—C—H. Mulliken has discussed delocalization in ethane, calling it "hyperconjugation."[3] We believe that straightness of orbital path will contribute to bonding. For different ethane configurations, a given H—C—C—H path will have different shapes. The path will be "smoothest" when the hydrogen atoms are trans, so that delocalization bonding will be best for this configuration. The staggered form of ethane has three such smooth paths and will therefore be triply stabilized by this effect. Since the earliest calculations[4] it has seemed clear that steric effects could not explain the ethane barrier but could account for only about a 0.3 kcal/mole contribution. If the ethane barrier is 3.0 kcal/mole, it would appear that the H—C—C—H six-electron delocalization bond has a strength of

about 0.9 kcal/mole when the hydrogens are trans (assuming negligible delocalization bonding in other configurations).

Another interesting example (called to our attention by Professor R. E. Powell) is the *trans* effect of Chernyaev.[5] A group *trans* to an electronegative or other labilizing group is weakened in its attachment relative to *cis* groups. As we have said above in a different way, an electronic orbital behaves like a fluid of high surface tension.[6] Thus we are led to an interpretation of such phenomena as the higher dissociation constants of the *trans* forms of compounds such as $Pt(NH_3)_2(OH)_2$ and $[Pt(NH_3)_2(H_2O)_2]^{++}$. The potential "hole" left by the removal of one of the negative groups allows the "fluid" orbital to flow into the vacancy. The square planar configuration is such that there is less orbital curvature when the potential hole is *trans* than when it is *cis*, thereby stabilizing the *trans* dissociation product to a greater extent than the *cis* product.

In this light, the *trans* effect is attributed, not to the labilizing of the *trans* bonds, but rather to a greater stabilizing of the activated complex in solvolysis as *trans* elimination proceeds, as compared with *cis* elimination.

The *trans* effect is not restricted to the metal chelates. The same principles can be applied to the elimination reactions of organic chemistry, which usually involve elements which are *trans* to each other. Effect 3 also makes readily understandable the usually greater stability of *trans*- over *cis*-disubstituted ethylenes, planarity of hydrocarbon chains, the greater stability of the chair form of cyclohexane, and other phenomena. A more detailed discussion is in preparation.

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*THE PATH OF CARBON IN PHOTOSYNTHESIS. XXII. THE IDENTIFICATION OF CARBOXY-KETOPENTITOL DIPHOSPHATES AS PRODUCTS OF PHOTOSYNTHESIS**

BY V. MOSES† AND M. CALVIN

RADIATION LABORATORY AND THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY

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INTRODUCTION

It has been well established that the initial carboxylation reaction of photosynthesis in green plants is the combination of carbon dioxide with ribulose 1,5-diphosphate followed by hydrolysis of the product to form two molecules of 3-phosphoglyceric acid.[1–5] Calvin[6, 7] has suggested that the reaction proceeds via a six-carbon sugar keto acid diphosphate intermediate, 2-carboxy-3-ketopentitol-1,5-

diphosphate (2-phosphohydroxymethyl-3-ketopentonic acid-5-phosphate), which has, however, not been identified in experiments involving photosynthesis in the presence of radioactive carbon dioxide, hitherto reported.

The standard methods used in this laboratory for the analysis of the products of short-term photosynthesis experiments performed in the presence of $C^{14}O_2$ involve killing the cells at the desired time by rapidly pouring the cell suspension into four volumes of boiling ethanol. The cell residue is then centrifuged and re-extracted with boiling 20 per cent ethanol, followed by a final extraction with water. The pooled extracts are evaporated to a small volume *in vacuo* at temperatures not exceeding 40°, and aliquots are chromatographed on sheets of oxalic acid–washed Whatman No. 4 filter paper. The solvents (phenol-water in the first dimension and butanol–propionic acid–water in the second dimension) are normally allowed to run only to the bottom edge of the paper (8–10 hours at 25°), or at most twice that far, before the papers are dried. The locations of radioactive substances on the chromatograms are determined by radioautography.[8]

These techniques, while very valuable for general purposes, suffer from at least two disadvantages. First, the extraction of the cells by boiling ethanol-water mixtures is liable to cause breakdown of heat-labile substances, of which 2-carboxy-3-ketopentitol-1,5-diphosphate (being a β-keto acid) may be expected to be one. Second, on chromatograms run for the standard lengths of time, the area near the origin containing principally the sugar phosphates, and particularly the sugar diphosphates, is not well separated; it is known that the apparently single spots seen in this area are often mixtures containing several diphosphate esters of the relevant sugars.[9]

The present communication reports attempts which have been made to overcome both these difficulties in an effort to isolate and identify the intermediates proposed for the reaction between ribulose diphosphate and carbon dioxide to form phosphoglyceric acid. Two substances of the carboxy-ketopentitol diphosphate type have now been tentatively identified.

METHODS

Cells of *Chlorella pyrenoidosa* for experimental purposes were grown in the continuous culture apparatus described by Holm-Hansen *et al.*[10] The cells were harvested, washed twice with distilled water, and 400 μl. of wet-packed cells were resuspended in 10 ml. of distilled water in a "lollipop" of suitable size. To obtain the cells in a steady metabolic state, the cell suspension was flushed for 30 minutes with air containing 1 per cent (*v*/*v*) CO_2, the lollipop being immersed in a water bath at room temperature and illuminated from both sides with 150-watt RS-P-2 photospot lamps. After 30 minutes of flushing, the gas flushing tube was removed from the lollipop, and 1 minute later 0.25 ml. of $NaHC^{14}O_3$ (100 μC.; 6.6 μmoles) was added. The lollipop was stoppered and shaken, and the cells were allowed to photosynthesize between the light sources for 3 minutes. The cell suspension was then poured rapidly into 40 ml. of ethanol (precooled to −15°) and was extracted overnight at −15°. After centrifugation at this temperature, the residue was extracted overnight with 20 ml. of 20 per cent ethanol at 0° and again with 10 ml. of 20 per cent ethanol at 0° overnight. The cell residue was finally extracted with 10 ml. of 20

per cent ethanol at 60° for 30 minutes. The extracts were pooled and evaporated to a small volume *in vacuo* at room temperature.

Aliquots of the concentrated extract were spotted onto oxalic acid–washed Whatman No. 4 filter paper, using a current of air at room temperature to dry the spots; the chromatograms were developed in the first dimension with phenol-water and in the second dimension with *n*-butanol–propionic acid–water, the solvents being allowed to run for 48 hours in each direction. Radioactive materials were located by radioautography, using Dupont blue-sensitive single-coated X-ray film 507E.

RESULTS

Chromatography for 48 hours in each dimension of the ethanol- and water-soluble components of algal cells after 3 minutes of photosynthesis in the presence of $C^{14}O_2$ showed the presence of three distinct radioactive spots in the diphosphate area of the chromatogram (Fig. 1). Each of these spots was cut out, eluted, phos-

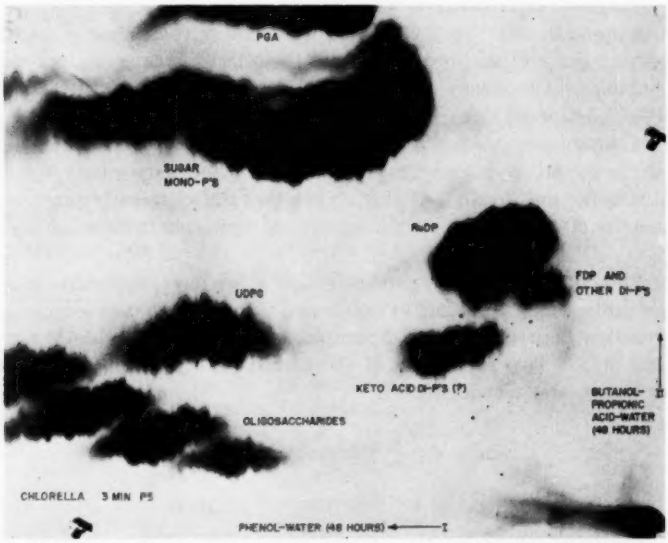


FIG. 1.—Radioautogram of ethanol-water extract of *Chlorella* after 3 minutes of photosynthesis in the presence of $C^{14}O_2$. The solvents were allowed to run for 48 hours in each dimension.

phatased, and rechromatographed. The spot labeled "RuDP" contained, after phosphatasing, predominantly ribulose, smaller quantities of glyceric and glycollic acids, together with twelve or more weakly radioactive substances which were not identified. The spot labeled "FDP and other Di-P's" contained, after phosphatasing, mainly fructose, glucose, and sedoheptulose, together with small quantities of glyceric and glycollic acids and one unidentified compound. The third spot ("Keto acid Di-P's"), never previously observed, ran more slowly in the butanol–propionic acid solvent than did the other two. It seemed possible that this spot might contain a component more acidic than the other sugar diphosphates and steps were taken to determine the nature of this substance.

After treatment with purified Polidase S[11] or with human seminal acid phosphatase[12] overnight in approximately 0.02 *M* acetate buffer, pH 5, a number of spots were seen on subsequent chromatography and radioautography (Fig. 2). Two of these appeared of particular interest, and they have now been tentatively identified (see "Discussion") as 2-carboxy-4-ketopentitol ($CH_2OH \cdot COH(COOH) \cdot CHOH \cdot CO \cdot CH_2OH$) and the lactone of 2-carboxy-3-ketopentitol ($CH_2OH \cdot COH(COOH) \cdot CO \cdot CHOH \cdot CH_2OH$). These substances will henceforth be referred to as the *γ-keto acid* and the *β-keto acid*, respectively.

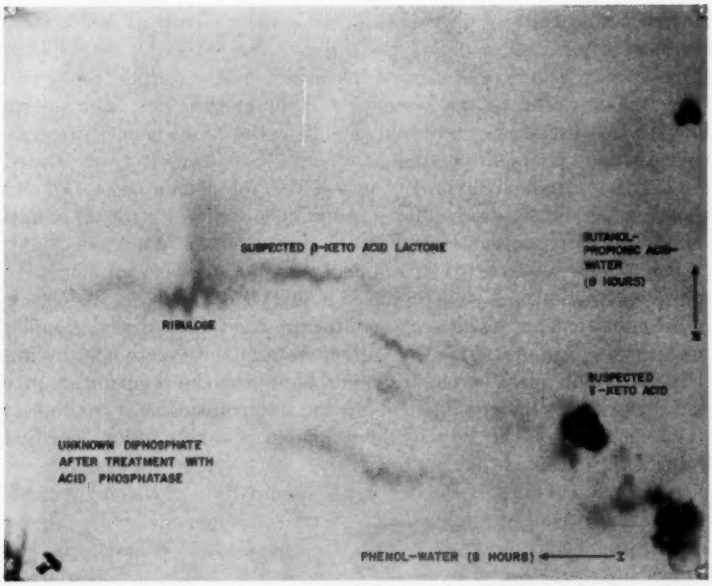


FIG. 2.—Radioautogram of spot labeled "Keto acid Di-P's" from Fig. 1, after phosphatasing.

Investigation of the γ-Keto Acid.—A number of tests have been applied to determine the structure of this compound, the results of which are consistent with its being 2-carboxy-4-ketopentitol. At no time have more than tracer amounts of either the β- or the γ-keto acid been available, and all investigations have depended on following the radioactivity after various treatments.

a) Electrophoresis and Chromatography with Known Substances.—Electrophoresis on oxalic acid–washed Whatman No. 4 filter paper in 0.1 *M* ammonium acetate buffer, pH 9, for 3 hours at 600–700 volts, together with authentic samples of gluconic acid, 2-ketogluconic acid, 5-ketogluconic acid, and hamamelonic acid, showed that all the available known six-carbon acids and the unknown substance ran the same distance along the paper and could be separated from five-carbon and seven-carbon sugar acids. The known six-carbon sugar acids could not be separated from each other or from the unknown substance when chromatographed in the solvent systems mentioned above, suggesting that the unknown had a generally similar structure, i.e., a polyhydroxymonocarboxylic six-carbon sugar acid.

b) Lactonization.—Treatment of the γ-keto acid with 0.07 *M* HCl at 100° for 30 minutes produced no lactone (Fig. 3), suggesting that it is not a simple aldonic acid. Polyhydroxymonocarboxylic sugar acids all readily lactonize in acid solution. However, the keto acids—2-ketoluconic acid and 5-ketoluconic acid—undergo direct acid lactonization with much greater difficulty than the parent hydroxy acid, gluconic acid.[13]

c) Enolization and Epimerization by Alkali.—The γ-keto acid was allowed to stand for 9 hours at room temperature at pH 11–12 (NaOH), and was then acidified at 0° with HCl to pH 3. Chromatography showed the formation, by such treatment, of some ribulose and a trace of glyceric acid, while most of the original material remained apparently intact (Fig. 4); there thus appeared to be no stable epimer formed by alkali. The double spot of the γ-keto acid resulting from this treatment was probably due to the presence of sodium chloride. The question of enolization will be discussed below in the section on the β-keto acid (paragraph *a*).

d) Treatment with 2,4-Dinitrophenylhydrazine.—The γ-keto acid was treated with an excess of 2,4-dinitrophenylhydrazine in 2 *M* HCl for 30 minutes at 37°. Carrier 5-ketogluconic acid was also present. The hydrazones were extracted from the reaction mixture and chromatographed in one dimension on Whatman No. 1 filter paper, using *n*-butanol saturated with 6 per cent (*w/v*) aqueous ammonia.[14] Two 2,4-dinitrophenylhydrazones were produced in low yield, which ran with the hydrazones of the added carrier. This suggested the presence of a carbonyl grouping.

e) Reduction with Borohydride.[15, 16]—Reduction of the γ-keto acid by approximately 0.0075 *M* potassium borohydride for 2 hours at room temperature produced a substance capable of undergoing acid-lactone interconversion at appropriate pH levels (Figs. 5 and 6). This implies the reduction of a keto group to a hydroxyl group, which is then capable of lactonization.

The product found after reduction with borohydride chromatographed very nearly, but not identically, with hamamelonic acid and hamamelonic lactone. Two samples of "hamamelonic acid" were available; one, made by Schmidt and Heintz,[17] was obtained by the action of hydrogen cyanide on *d*-ribulose, followed by hydrolysis of the nitrile and separation of the epimeric acids produced, by fractional crystallization of their phenylhydrazide derivatives.[18] The other sample prepared by Rabin *et al.*,[19] was synthesized by treating ribulose diphosphate with potassium cyanide, with simultaneous hydrolysis of the nitrile to hamamelonic acid diphosphate, followed by enzymatic removal of the phosphate groups. This sample, presumably, contained hamamelonic acid and its epimer in unknown proportions.

The γ-keto acid, after reduction with potassium borohydride, chromatographed more nearly with Rabin *et al*'s. sample of mixed hamamelonic acid and epimer (or their lactones) (Figs. 7 and 8) than with Schmidt and Heintz's pure hamamelonic acid or lactone (Figs. 5 and 6). Before reduction with borohydride, the γ-keto acid ran quite distinctly from the hamamelonic acid of Schmidt and Heintz (Fig. 9), and no lactonization could be observed (Fig. 3). The explanation for these findings may be that the γ-keto acid is reduced by borohydride to a mixture of two epimers of the hamamelonic acid type, neither of which is hamamelonic acid itself, but one of which is the same as the epimer of hamamelonic acid present in Rabin *et al*'s. sample.

f) Treatment with Acid.—On heating with 0.07 *M* HCl for 30 minutes at 100°, some 35–45 per cent of the γ-keto acid was converted into xylulose, presumably by

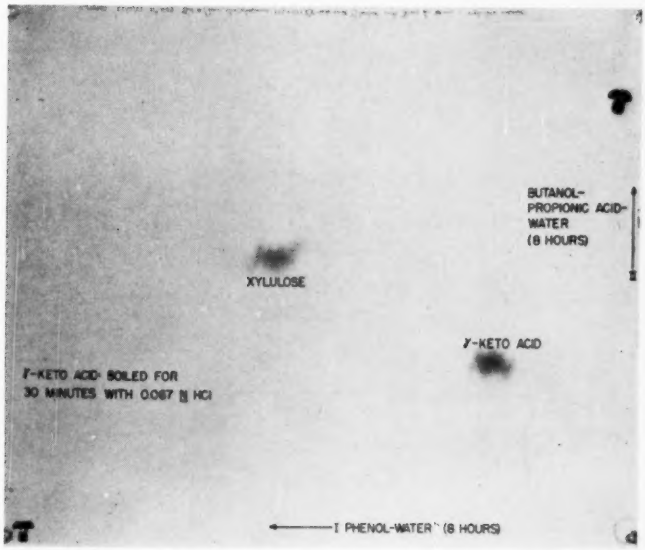


FIG. 3.—Radioautogram of γ-keto acid after treatment with 0.07 *M* HCl for 30 minutes at 100°.

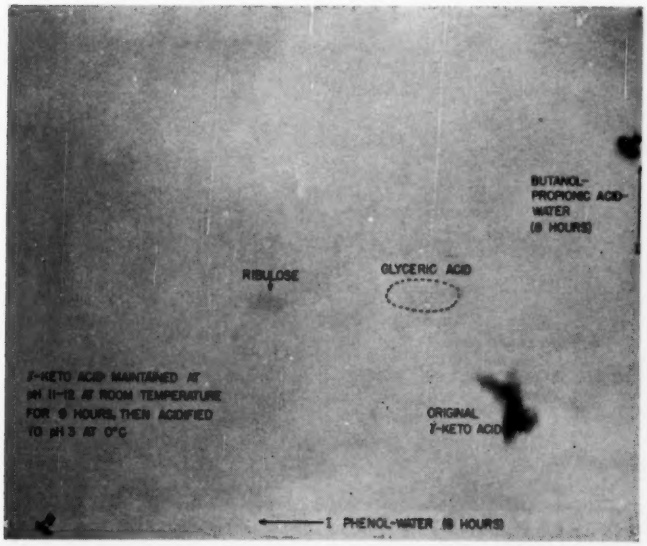


FIG. 4.—Radioautogram of the γ-keto acid after being maintained at pH 11–12 at room temperature for 9 hours, followed by acidification to pH 3 at 0°.

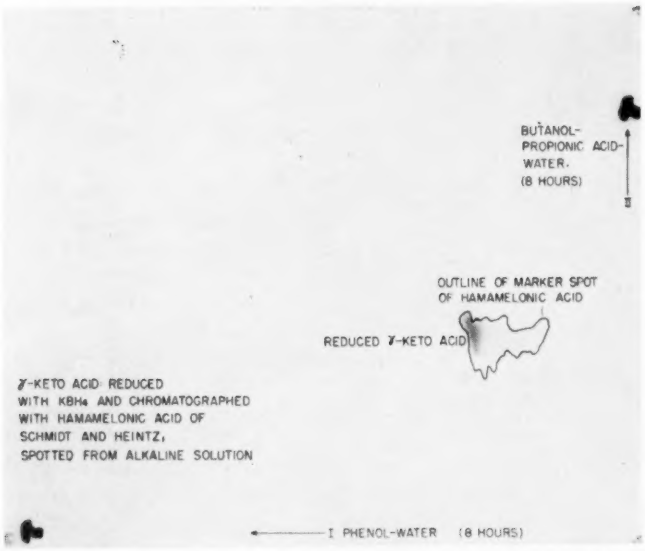


FIG. 5.—Radioautogram of the γ-keto acid, reduced with potassium borohydride and co-chromatographed with the hamamelonic acid sample of Schmidt and Heintz (*Ann.*, **515**, 77, 1935), spotted from alkaline solution (pH 13). The outline of the marker spot of authentic hamamelonic acid has been drawn in.

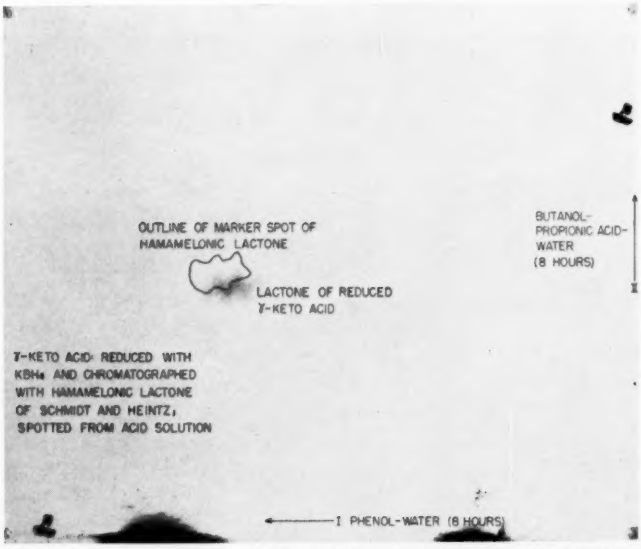


FIG. 6.—Radioautogram of the γ-keto acid, reduced with potassium borohydride, and co-chromatographed with the hamamelonic acid sample of Schmidt and Heintz [*Ann.*, **515**, 77, (1935)], spotted from acid solution (pH 1). The outline of the marker spot of authentic hamamelonic lactone has been drawn in.

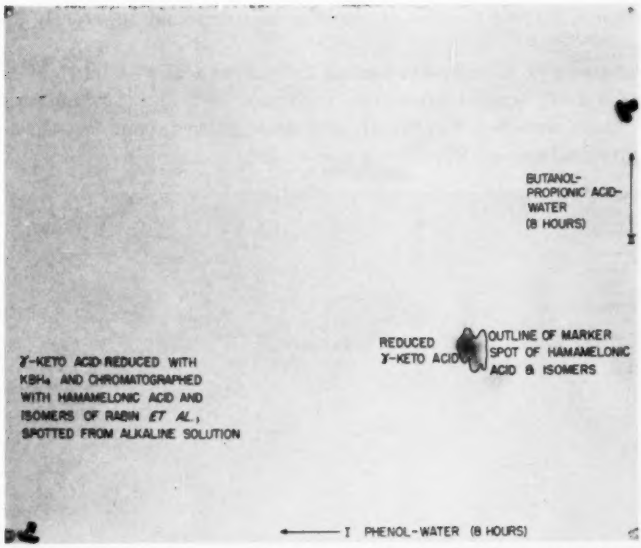


FIG. 7.—Radioautogram of the γ-keto acid, reduced with potassium borohydride and co-chromatographed with the hamamelonic acid sample of Rabin *et al.* (*J. Am. Chem. Soc.* [in press]), spotted from alkaline solution (pH 13). The outline of the marker spot of authentic hamamelonic acid (and epimer) has been drawn in.

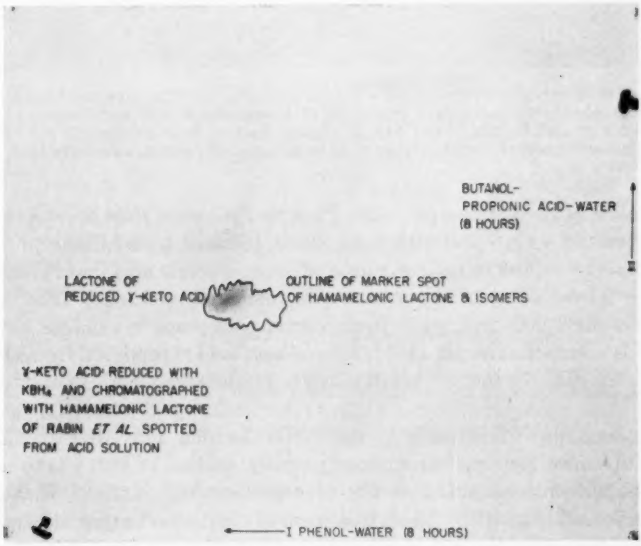


FIG. 8.—Radioautogram of the γ-keto acid, reduced with potassium borohydride and co-chromatographed with the hamamelonic acid sample of Rabin *et al.* (*J. Am. Chem. Soc.* [in press]), spotted from acid solution (pH 1). The outline of the marker spot of authentic hamamelonic lactone (and epimer) has been drawn in.

decarboxylation, leaving the rest of the original compound apparently unchanged (Fig. 3).

g) Treatment with Alkali.—On heating the γ-keto acid with 0.015 *M* NaOH for 30 minutes at 100°, several substances were produced, one of which was glyceric acid (the others were not identified), and again some (about two-thirds) of the original compound was left apparently unchanged.

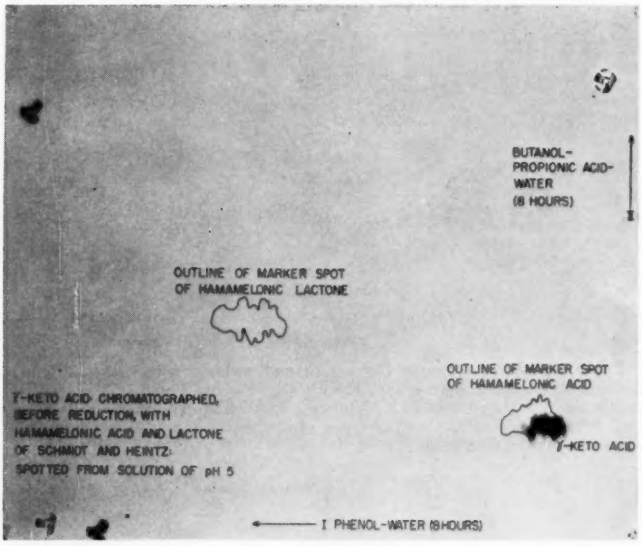


FIG. 9.—Radioautogram of the γ-keto acid prior to reduction, co-chromatographed with the authentic sample of hamamelonic acid and lactone of Schmidt and Heintz (*Ann.*, **515**, 77, 1935), spotted from solution of pH 5. The outlines of the marker spots of hamamelonic acid and lactone have been drawn in.

Investigation of the β-Keto Acid. a) Possible Formation from the γ-Keto Acid.—Treatment of the γ-keto acid with weak alkali, followed by acidification (see paragraph *c* above) resulted in the formation of some glyceric acid and ribulose, while most (over 90 per cent) of the γ-keto acid remained unchanged (Fig. 4). Acid treatment of the γ-keto acid results in the partial conversion to xylulose, not ribulose (Fig. 3). It seems that at pH 11–12, some β-keto acid is produced from the γ-keto acid by enolization, the former decomposing to glyceric acid in alkali and to ribulose in acid.

b) Lactonization.—Treatment of the β-keto lactone with 10^{-4} *M* NaOH produced a substance running chromatographically similar to the γ-keto acid (i.e., just behind hamamelonic acid; see Fig. 9), together with a little of what appeared to be glyceric acid (Fig. 10). Alkali treatment of the β-keto lactone apparently converted it partly into the β-keto acid and also caused some breakdown to glyceric acid (cf. paragraph *g* above).

c) Reduction with Borohydride.—When the β-keto lactone was treated with 0.0075 *M* potassium borohydride for 2 hours at room temperature (pH about 10),

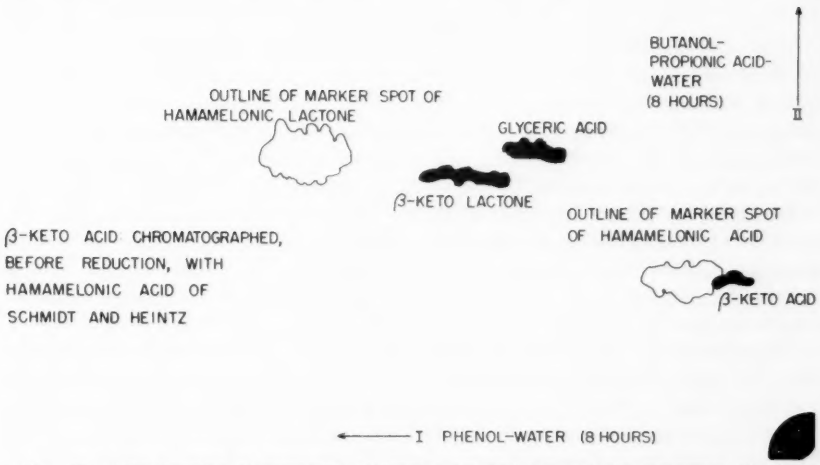


FIG. 10.—Line drawing of composite radioautogram and sprayed chromatogram of β-keto lactone co-chromatographed with hamamelonic acid and lactone of Schmidt and Heintz (*Ann.*, **515,** 77, 1935), spotted from a solution of pH 10. Solid areas represent radioactive substances; outlines are of authentic compounds.

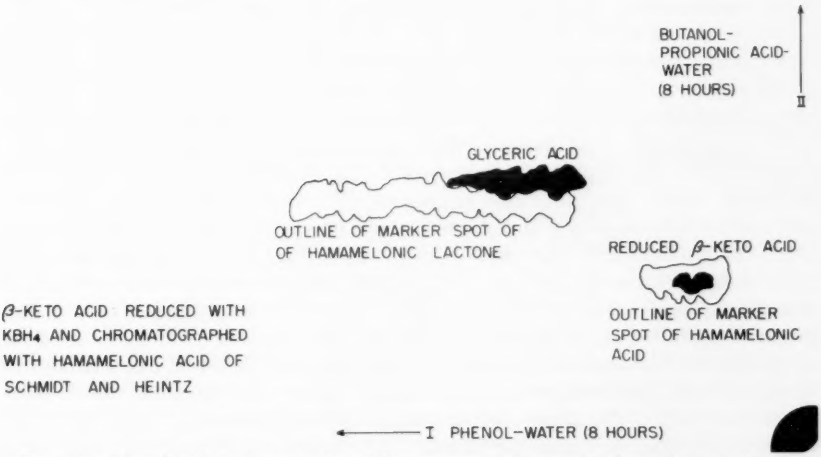


FIG. 11.—Line drawing of composite radioautogram and sprayed chromatogram of β-keto lactone after reduction with potassium borohydride and chromatographed with hamamelonic acid and lactone of Schmidt and Heintz (*Ann.*, **515,** 77, 1935), spotted from solution of pH 10. Solid areas represent radioactive substances; outlines are of authentic compounds.

apparently some glyceric acid and a substance which ran chromatographically together with hamamelonic acid were formed (Fig. 11). However, the radioactivity was too weak to determine the exactitude of coincidence with hamamelonic acid.

A careful investigation of each of the three diphosphate spots shown in Figure 1 has not revealed the presence of any other substances either of this type (keto acid) or of the reduced form (hamamelonic acid and epimers).

DISCUSSION

While the final identification of the two materials under investigation must await co-chromatography with authentic samples, the evidence accumulated here permits a tentative assignment of structure, with some degree of confidence. The position of the original spot on the chromatogram (Fig. 1) is fairly definite evidence of its diphosphate character. Its susceptibility to the enzyme phosphatase, resulting in a material apparently free from phosphate, may be taken as additional evidence for its phosphate ester character. Most of the evidence available has been obtained with the more plentiful and the more stable of the two new compounds, and the course of the argument for the structure of this compound will first be outlined, followed by inferences from this substance and additional evidence for the structure of the second and more unstable compound.

The chromatographic and electrophoretic behavior of the dephosphorylated material seems to establish its character as a hydroxy sugar acid; beyond this, its location in the region of gluconic acid is fairly definite evidence of its weight, corresponding to that of a six-carbon polyhydroxy acid. While the possibility of a heptonic acid is not eliminated on chromatographic grounds alone, the other evidence to be mentioned later appears to preclude this possibility. Electrophoretically, the material moves exactly parallel with gluconic acid as well as with hamamelonic, 5-ketogluconic, and 2-ketogluconic acids. The demonstrated ability of paper electrophoresis to separate the pentonic acids from a variety of penta-oxyhexonic acids makes the value of the mobility of the unknown substance a significant quantity in establishing its penta-oxyhexonic character.

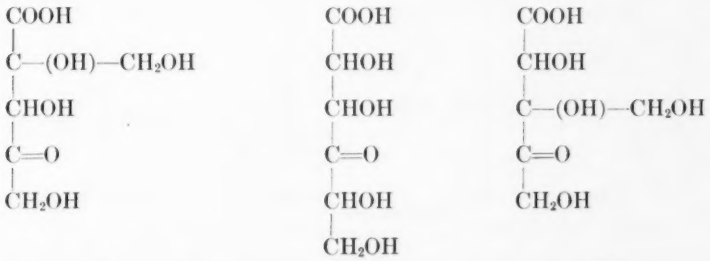
The failure of the compound to lactonize under acid conditions which lead to lactone formation in all other simple aldonic acids, such as gluconic, hamamelonic, ribonic, and similar acids, indicates that the material is not a simple aldonic acid. Reduction by potassium borohydride produces a material which does exhibit the facile acid lactone interconversion so characteristic of the aldonic acids. This information, taken together with the fact that the two keto-aldonic acids available to us—2-ketogluconic and 5-ketogluconic—are also sluggish in their lactonization under the conditions used in this work, suggests that the compound is originally a keto-aldonic acid. The co-chromatography of the reduced material, both in the free acid and in the lactone form, with hamamelonic acid and its lactone is so close as to confirm the idea that the material after reduction is indeed a six-carbon aldonic acid and therefore, before reduction, a six-carbon keto-aldonic acid. Co-chromatography of the unreduced substance with hamamelonic acid clearly demonstrated its difference from the latter. The treatment of the original keto acid with 0.07 *M* HCl for 30 minutes at 100° yielded about 40 per cent conversion to xylulose, the remaining 60 per cent being recovered as the original acid. This stability eliminates the serious possibility that the unknown substance is a β-keto acid.

The nearest analogy available to us is β-ketogluconic acid, which has been postulated as an intermediate in the conversion of 6-phosphogluconic acid to ribulose 5-phosphate.[20,21] The inability to isolate a sample of this material (6-phospho-3-ketogluconate) has been taken as evidence of its gross instability. The chemical analogies about which we do have direct information include acetoacetic acid, α,α-dimethylacetoacetic acid, and dihydroxymaleic acid (α-hydroxyoxaloacetic acid).[22]

The substitution of either methyl or of hydroxyl on the α-carbon atom of a β-keto acid increases its lability considerably. Neither of the latter two acids could survive the 100° treatment for more than a few seconds to minutes. We can therefore be confident that the substitution of both methyl and hydroxyl on an α-carbon atom of a β-keto acid such as is here proposed would lead to an extremely labile compound. The possibility that we might be dealing with a 2-keto-3-carboxy pentitol stabilized against decarboxylation by a 2,5-ketol (furanoside) formation is eliminated on the grounds that such a furanoside would not exhibit the great susceptibility to borohydride reduction that this compound does. Beyond this, the appearance of glyceric acid in the degradation of the compound speaks against the carboxyl group being on the three-carbon atom of a five-carbon chain.

The clean and quantitative formation of xylulose under the very mild conditions of acid treatment described above eliminates both the α- and the δ-keto acids as possibilities since these acids would be unchanged under the mild conditions used here.[13] 2-Ketogluconic and 5-ketogluconic acids were treated with 0.07 *M* HCl for 30 minutes at 100° and chromatographed in the usual manner: they were found to be unchanged, and no neutral material (lactones or sugars) appeared. In stronger acid (5 *M* HCl) both the 2- and the 5-ketogluconic acid can be converted via the corresponding ascorbic acids to decarboxylation products such as furfural.[13] The likelihood thus remains that the compound is a 4-(or γ-)keto acid.

Steric considerations apart, three six-carbon γ-keto sugar acids are possible (I, II, and III):



I II III

Structure III can probably be excluded on the grounds that decarboxylation of such a branched-chain sugar acid, if it occurred at all, would most probably result in a branched-chain pentose, or a tetrose, whereas xylulose was actually the decarboxylation product. Structures I and II cannot be unequivocally distinguished, though the available evidence favors I. Decarboxylation of II would be expected to be more difficult than that of I, since it would require the elimination of a secondary hydroxyl group in II rather than a tertiary one, as in I. Furthermore, the alkaline decomposition of the unknown substance (presumably via epimerization to the β-keto acid) to produce some glyceric acid is more in keeping with I than with II.

We must now provide a route for the formation of xylulose (IX) from such a γ-keto acid (IV) under the conditions in which we know this transformation occurs. Unfortunately, we have no single model compound incorporating all the structural features of this one, viz., an α-tertiary hydroxyl group, a β-hydroxyl group, and a γ-ketone function, which seem pertinent to an understanding of this transformation.

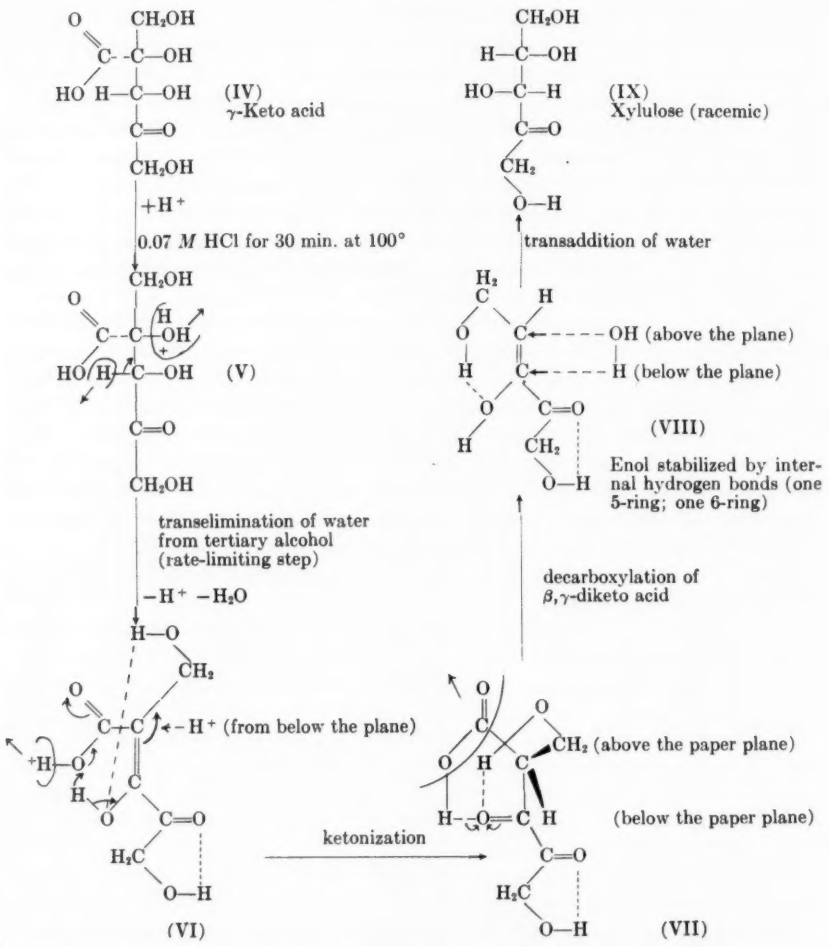


FIG. 12.—Proposed mechanism for the decarboxylation of the γ-keto acid to xylulose.

One can, however, formulate it in terms of a sequence of three reactions, all of which are known to take place rapidly under the conditions here required. These are (1) the transelimination of water from V to form the α,β-unsaturated, β-hydroxy, γ-keto acid (VI), which ketonizes to form the β,γ-diketo acid (VII); (2) the decarboxylation of this β-keto acid to form an enolic ketone (VIII); and (3) the transaddition of water to this enolic ketone, yielding xylulose (racemic)(IX)-(Fig. 12). The rates of all these reactions in separate model cases are known to be sufficiently rapid to account for this sequence. The absence of any other intermediates on the chromatogram (Fig. 3) would require that the over-all rate-limiting step be the transelimination of water (V to VI). The uncertainty lies in the possible simultaneous occurrence of the decarboxylation (VII to VIII) and the trans-

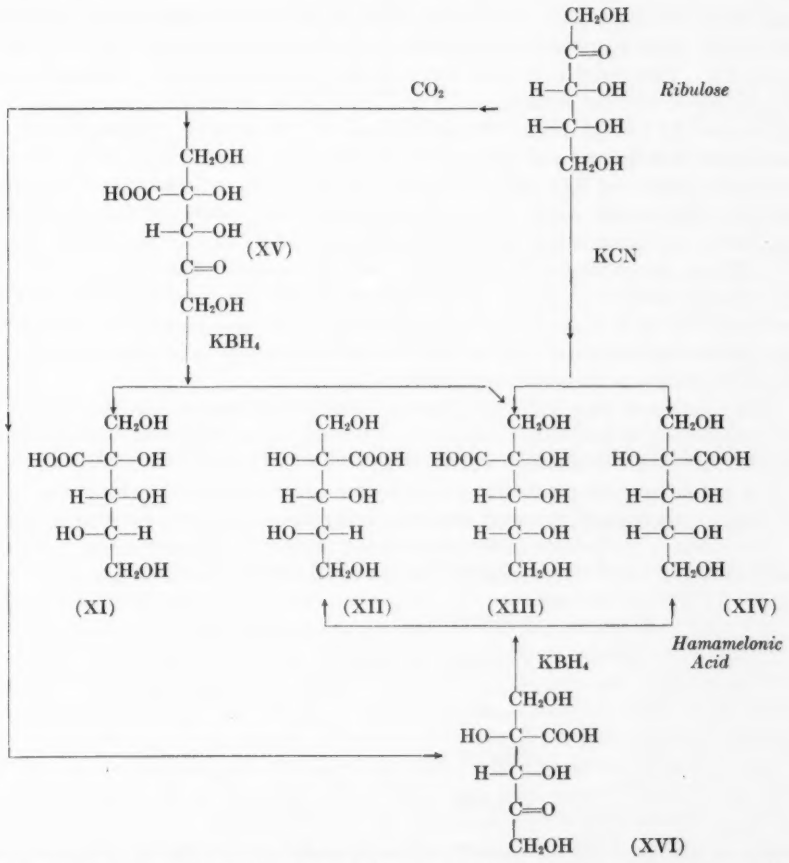


FIG. 13.—The steric relationships between the two possible diastereomers of the γ-keto acid and its reduction products, epimers of hamamelonic acid.

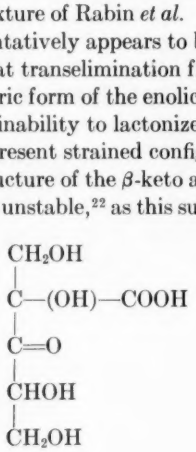
addition of water (VIII to IX). One can rationalize a stabilized enolic ketone (VIII) in terms of internal hydrogen bonds which would give it a lifetime long enough to provide the configurational requirement needed for the transaddition to produce xylulose. The possibility that the series of transformations may begin by the formation of the β,γ-enol, γ-lactone of IV is recognized and considered unlikely in view of the ease of the tertiary hydroxyl elimination to form VI.

The question of the steric configuration of the γ-keto acid poses a further consideration. Figures 5–8 show that, after reduction with borohydride, the γ-keto acid co-chromatographs very closely, though not identically, with the two samples of hamamelonic acid available. Figure 13 shows the steric relationship between the two possible diastereomers of the γ-keto acid (XV and XVI) and its reduction products, epimers of hamamelonic acid (XI to XIV). The sample of hamamelonic acid obtained by Schmidt and Heintz[17] contained only one isomer, hamamelonic

acid itself, structure XIV, while that of Rabin *et al.*,[19] prepared by the action of potassium cyanide on ribulose diphosphate, presumably contained structures XIII and XIV. Two possible epimeric forms of the γ-keto acid can be produced by carboxylation of ribulose, structures XV and XVI, which on reduction would result in substances XI and XIII and XV, and XII and XIV from XVI. Experimentally it was found that the reduced γ-keto acid co-chromatographed more closely with the mixture of XIII and XIV than with pure XIV itself. It could be argued from this that reduction of the γ-keto acid produced XI and XIII, neither of which was identical with the hamamelonic acid of Schmidt and Heintz (XIV)[17] and which therefore did not co-chromatograph precisely with this sample. However, one form of the reduced γ-keto acid (XIII) would correspond with one epimer in Rabin *et al*'s.[19] sample. The lack of exact co-chromatography in this case would then result from the presence of radioactive XI derived from the γ-keto acid by reduction and of carrier XIV present in the mixture of Rabin *et al.*

The γ-keto acid thus tentatively appears to be best represented by structure XV. It is interesting to note that transelimination from this stereoisomer would produce directly the proper geometric form of the enolic ketone VI (Fig. 12). The structure XV is consistent with the inability to lactonize, as six-membered lactone rings containing a ketone group represent strained configurations and tend not to be formed.

The evidence for the structure of the β-keto acid (XVII) is less extensive. β-Keto acids are known to be very unstable,[22] as this substance has been found to be



XVII

while the ability of the substance to lactonize under acid conditions (indeed, it was first observed as a lactone) would, in this case, involve a five-membered lactone ring containing a ketone group, which is a less sterically strained configuration. Under mild alkaline conditions the lactone ring is easily opened to produce the free acid, and the substance readily decomposes to form glyceric acid, by hydrolysis between carbon atoms 2 and 3. The free acid, like the γ-keto acid, does not co-chromatograph with hamamelonic acid, though, after reduction with borohydride, co-chromatography is very close to and perhaps identical with hamamelonic acid (Figs. 10 and 11).

Decarboxylation of the β-keto acid was not directly demonstrated, since the entire supply was used in the reduction experiment. However, treatment of the γ-keto acid with mild alkali, followed by acidification at 0° to pH 3, conditions which might be expected to produce some of the β-keto acid from the γ-keto acid by enolization, produced instead ribulose and glyceric acid (Fig. 4). The latter could result from hydrolytic cleavage of the β-keto acid at alkaline pH, and the ribulose by decarboxylation at acid pH. In this case decarboxylation would involve the migration of the carbonyl group (Fig. 14). No evidence is available as to the steric con-

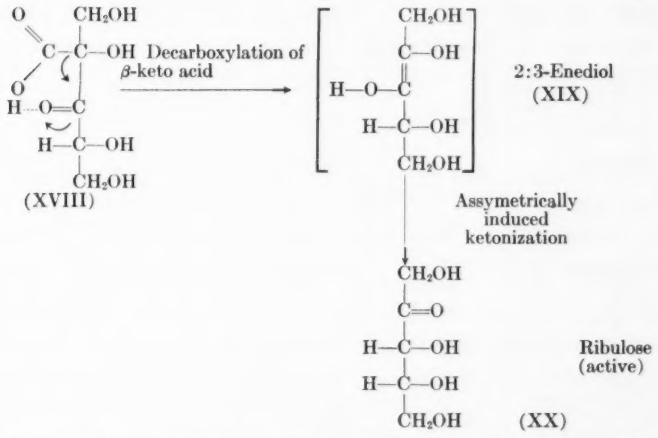
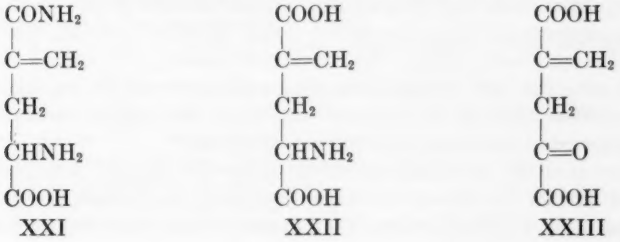


FIG. 14.—Suggested mechanism for the decarboxylation of the β-keto acid to ribulose.

figuration of the β-keto acid, save that the δ-carbon atom should have the same configuration as the original ribulose from which it was formed.

Biological Significance.—While the β-keto acid diphosphate has been predicted theoretically in the photosynthetic carboxylation of ribulose diphosphate,[6, 7] there has been, to our knowledge, no previous demand for the existence of the γ-keto acid, and hence no role is immediately apparent for it. It may, indeed, be an artifact resulting from the procedures of killing and extracting the cells and from the subsequent chromatography and thus be produced non-enzymatically from the β-keto acid or from another compound. However, it seems that, using identical killing and chemical techniques, more radioactive γ-keto acid appears to be formed from labeled carbon dioxide in cells which have been allowed to carry on photosynthesis in the presence of 0.001 *M* NH_4NO_3 than in cells suspended in distilled water. The γ-keto acid may accordingly be involved in nitrogen metabolism.

It is of interest in this connection to recall the findings of Done and Fowden,[23] who observed that in peanuts the most important free amide present in these plants was γ-methylene-glutamine (XXI), while the relative amount of glutamine in relation to asparagine was very much reduced. γ-Methylene-glutamic acid (XXII) and γ-methylene-α-ketoglutaric acid (XXIII) were also subsequently found in peanuts,[24, 25] and it was suggested[24] that these compounds may be the ones most intimately concerned with nitrogen transport in these plants. The similarity be-



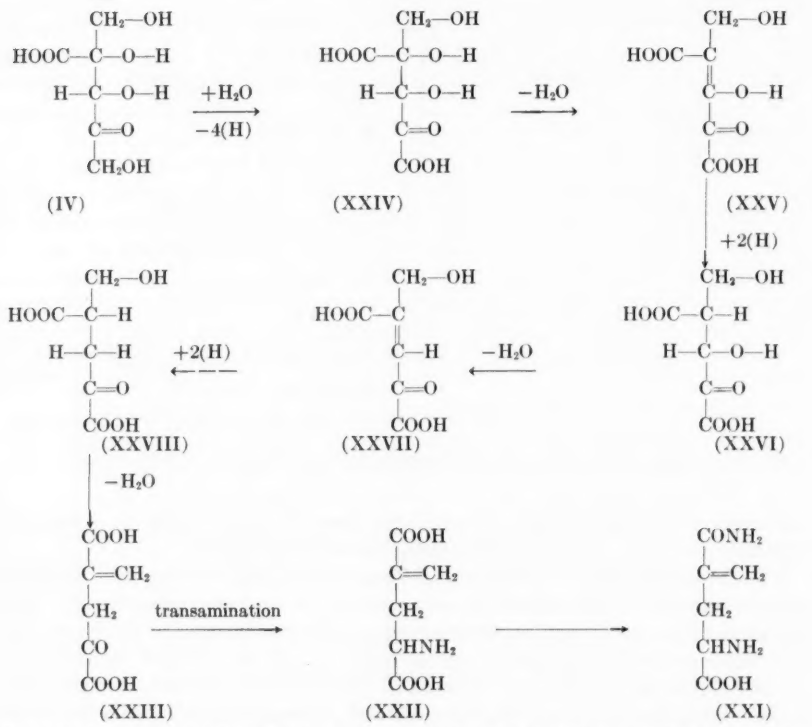


FIG. 15.—Hypothetical scheme for the conversion of the γ-keto acid into γ-methyleneglutamic acid.

tween the skeletal structures of the γ-keto acid (IV) and γ-methylene-glutamic acid and amide (XXI and XXII) are immediately apparent, and it would not be difficult to conceive a metabolic pathway for the production of γ-methylene-glutamic acid from the γ-keto acid. Such a hypothetical pathway, involving alternate dehydration and reduction following an initial oxidation, is illustrated in Figure 15, though many other schemes would be possible; it is of interest to note that the γ-keto acid and γ-methylene α-ketoglutaric acid are isoximeric.

Another possible route for the further metabolism of the γ-keto acid might be an enzymatic inverse aldolase split to hydroxypyruvate and dihydroxyacetone. The hydroxypyruvate could give rise by transamination to serine, a substance known to incorporate radiocarbon very rapidly from carbon dioxide during photosynthesis. The dihydroxyacetone, during the initial stages of photosynthesis, would not incorporate isotopic carbon from labeled carbon dioxide, and such a dilution of labeled dihydroxyacetone, formed from 3-phosphoglyceric acid, by this unlabeled dihydroxyacetone might in part account for some of the anomalous labeling which has been reported in the hexoses during photosynthesis.[26]

Treatment of all the three diphosphate spots shown in Figure 1 with phosphatase, followed by further chromatography, has confirmed the findings of Rabin *et al*,[19] that hamamelonic acid diphosphate is not formed during photosynthesis in the ab-

sence of inhibitors. Apart from the β- and γ-keto acids which form the subject of this communication, no other acids moving chromatographically in the area of hamamelonic acid or its lactone have been found in any of the three diphosphate spots of the original chromatogram.

SUMMARY

Two new compounds, tentatively identified, have been isolated from chromatograms of ethanol-water extracts of cells of *Chlorella pyrenoidosa* which had been allowed to carry on photosynthesis for 3 minutes in the presence of $NaHC^{14}O_3$. Chemical investigation suggested that the two compounds were 1,5 diphosphate esters of 2-carboxy-4-ketopentitol and of the lactone of 2-carboxy-3-ketopentitol. The latter has been postulated as an intermediate in the photosynthetic carboxylation of ribulose-1,5-diphosphate to yield two molecules of 3-phosphoglyceric acid. While the former might be an artifact resulting from the techniques used to kill, extract, and analyze the cells, possible biological roles for it have been suggested.

* The work described in this paper was sponsored in part by the U. S. Atomic Energy Commission and in part by the Department of Chemistry, University of California, Berkeley, California.

† Postgraduate Traveling Fellow of the University of London, 1956–57.

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MEASURES ORTHOGONAL TO POLYNOMIALS

By Errett Bishop*

UNIVERSITY OF CALIFORNIA

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The structure of a (finite, complex-valued, Borel) measure on the unit circle $|w| = 1$ which is orthogonal to all polynomials in w is well known. It is given by the following theorem,[1] which is partially due to F. and M. Riesz, and partially due to Fatou.

THEOREM 0. *If ν is a measure on $|w| = 1$ which is orthogonal to all polynomials, then ν is absolutely continuous with respect to Lebesgue measure dw on $|w| = 1$. The analytic function $f(w) = \frac{1}{2\pi i}\int (t - w)^{-1}\, d\nu(t)$ on $|w| < 1$ has radial boundary values equal to $\frac{d\nu}{dw} = v(w)$ at almost all points on the unit circle. The function $f(re^{i\theta})$ of θ. converges in mean to $v(e^{i\theta})$ as $r \to 1$.*

The purpose of this paper is to extend this theorem. Let C be a compact subset of the complex plane, with connected interior U and connected complement. Let μ be a measure on the boundary B of C which is orthogonal to all polynomials. An analytic differential $g(z)dz$ on U will be said to represent μ if there exists a sequence $\{\gamma_n\}$ of simple closed rectifiable curves lying in U for which $[g, \gamma_n] \to \mu$ as $n \to \infty$, in the weak star topology for measures on C, where $[g, \gamma_n]$ is that measure on γ_n obtained by integrating $g(z)dz$. It is clear that $g(z)dz$ will represent some measure on B if and only if a sequence $\{\gamma_n\}$ converging to B can be found for which the norms $\|[g, \gamma_n]\|$ are bounded. Thus, if φ is a conformal map of U onto the interior of another set such as C, the differential $\varphi(g(z)dz) = g(\varphi^{-1}(w))d\varphi^{-1}(w)$ on $\varphi(U)$ will represent a measure on the boundary of $\varphi(U)$ if and only if $g(z)dz$ represents a measure on B. Also, if $g(z)dz$ represents μ, then $g(z) = \lim_{n\to\infty} \frac{1}{2\pi i}\int_{\gamma_n} (\zeta - z)^{-1}$ $g(\zeta)d\zeta$, so that

$$g(z) = \frac{1}{2\pi i}\int (\zeta - z)^{-1}\, d\mu(\zeta). \tag{1}$$

THEOREM 1. *If φ is a conformal map of U onto $|w| < 1$, if μ is a measure on B which is orthogonal to all polynomials, and if $g(z)$ is defined by (1), then there exists a measure ν on $|w| = 1$ which is orthogonal to all polynomials, such that the differential $f(w)dw = \varphi(g(z)dz) = g(\varphi^{-1}(w))d\varphi^{-1}(w)$ on $|w| < 1$ represents ν, and such that all statements of Theorem 0 hold for ν and f.*

Proof: Let $\{U_n\}$ be a sequence of simply connected open sets containing C, with $U_{n+1} \subset U_n$ and $\cap U_n = C$. Let z_0 be a point in U, and let φ_n be the conformal map of U onto $|w| < 1$, with $\varphi_n(z_0) = \varphi(z_0)$ and $\varphi_n'(z_0)$ $[\varphi'(z_0)]^{-1} > 0$. Then $\varphi_n \to \varphi$ uniformly on all compact subsets of U as $n \to \infty$.[2] From this it is not hard to show that

$$f(w) = \lim_{n\to\infty} \frac{1}{2\pi i} \int [\zeta - \varphi_n^{-1}(w)]^{-1}\, d\mu(\zeta)\, \frac{d}{dw}(\varphi_n^{-1}(w)) =$$

$$\lim_{n\to\infty} \frac{1}{2\pi i} \int [t - w]^{-1}\, d[\varphi_n(\mu)]\,(t)$$

Since the measures $\varphi_n(\mu)$ all have norms equal to $\|\mu\|$, there will exist a subsequence of these measures which will converge to a measure ν on $|w| = 1$ in the weak star topology. By the last equation, $f(w) = \frac{1}{2\pi i}\int (t - w)^{-1}\, d\nu(t)$. Thus all statements of Theorem 0 obtain. In particular, $f(w)dw$ represents ν (take γ_n to be $|w| = 1 - \frac{1}{n}$).

THEOREM 2. *The differential $g(z)dz$ represents μ.*

Proof: Since the differential $f(w)dw$ defined in Theorem 1 represents ν, it follows that $g(z)dz$ represents some measure λ on B. Thus $\int (z - \zeta)^{-1}\, d(\mu - \lambda)\,(\zeta) = 2\pi i(g(z) - g(z)) = 0$, for all z in U. Since μ and λ are orthogonal to all polynomials, $\int (z - \zeta)^{-1}\, d(\mu - \lambda)\,(\zeta) = 0$ for all z in the complement of C. Hence $\mu - \lambda$ is orthogonal to all rational functions whose poles are in the complement of B. It is known[3] that any continuous function on B can be uniformly approximated by such rational functions. Thus $\mu - \lambda$ is orthogonal to all continuous functions on B, so that $\lambda = \mu$, thus proving the theorem.

By a theorem of Fatou,[2] the mapping function φ^{-1} will have radial boundary values $u(w)$ at almost all points w of the unit circle, and clearly $u(w) \in B$. It is known[2] that distinct points have distinct radial boundary values, so that u is one to one. By Lusin's theorem, u will be a homeomorphism on arbitrarily large closed subsets of $|w| = 1$, so that there will exist a Borel function u^{-1} on B with $u^{-1}(u(w)) = w$, a.e. on $|w| = 1$. Let ν_0 be the measure dw on $|w| = 1$. Since ν_0 is represented by the differential dw on $|w| < 1$, the differential $d\varphi(w)$ on U represents some measure μ_0 on B.

THEOREM 3. *The following statements are true:*

(i) $\mu = u(\nu)$.

(ii) $\nu = u^{-1}(\mu)$.

(iii) *μ is absolutely continuous with respect to μ_0 and* $\frac{d\mu}{d\mu_0} = v \circ u^{-1}$.

Proof: Let h be any continuous function on C. We can choose a sequence $\{r_n\}$ converging to 1, with $0 \leq r_n \leq 1$, such that $[g, \gamma_n]$ converges as $n \to \infty$ in the weak star topology to some measure λ on B, where $\gamma_n = \varphi^{-1}\{w \mid |w| = r_n\}$, since $\|[g, \gamma_n]\| = \|[f, |w| = r_n]\| \to \|\nu\|$ as $n \to \infty$ by Theorem 0. As in the proof of Theorem 2, $\lambda = \mu$. Thus, if h is any continuous function on C, we have

$$\int h\, d\mu = \lim_{n\to\infty} \int_{\gamma_n} h(z)g(z)dz = \lim_{n\to\infty} \int_{|w|=r_n} h(\varphi^{-1}(w))f(w)dw =$$

$$\int h(u(w))d\nu(w) = \int h du(\nu).$$

This proves (i). It follows that $u^{-1}(\mu) = u^{-1}(u(\nu)) = \nu$, proving (ii). To prove (iii), let h be any continuous function on C. Then

$$\int (v \circ u^{-1})h\, d\mu_0 = \int (v \circ u^{-1})h\, du(\nu_0) = \int (h \circ u)v\, d\nu_0 = \int (h \circ u)d\nu = \int h\, d\mu,$$

since (i) and (ii) are also valid for μ_0 and ν_0. This proves (iii).

As an application of Theorem 3, let μ be real. Then ν is real by (ii). Hence $\nu = 0$. Then $\mu = 0$ by (i). It follows that, for any continuous function h on B, there exists a sequence of polynomials whose real parts converge uniformly to h on B. By the maximum modulus principle, the sequence of real parts must converge uniformly on C, and the limit function will be harmonic on U. Thus we have:

THEOREM 4. *Let h be a continuous function on B. Then h can be extended to be continuous on C and harmonic on U, and the extended function can be uniformly approximated on C by real parts of polynomials.*

This theorem includes the solution of the Dirichlet problem for C, which is known. The further fact that the resulting harmonic extension of h can be uniformly approximated by real parts of polynomials seems to be new.

As an application of Theorem 4, one can prove the following theorem,[4] whose proof will be given elsewhere:

THEOREM 5. *If A is a uniformly closed algebra of continuous functions on B, which includes the polynomials, then either each function of A can be extended to be continuous on C and analytic on U, or A consists of all continuous functions on B.*

This generalizes a well-known result of Wermer.[5]

* This work was done with the support of ONR Contract NONR-222(37).

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NON-PARALLELIZABILITY OF THE n-SPHERE FOR n > 7

BY MICHEL A. KERVAIRE

INSTITUTE FOR ADVANCED STUDY

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We shall show that the recent results of R. Bott[1, 2]: $\pi_{2q}(\boldsymbol{U}(q)) \approx \boldsymbol{Z}/q!\boldsymbol{Z}$ and the periodicity of the stable homotopy groups of $\boldsymbol{SO}(n)$ and $\boldsymbol{U}(n)$ imply the

THEOREM. *For $s \geqq 3$, the sphere S_{4s-1} of dimension $4s - 1$ is not parallelizable.*

Recall that a differentiable closed manifold of dimension n is said to be *parallelizable* if it admits a continuous field of tangent n-frames. It is well known that S_{4s+1}, $s \geqq 1$, is not parallelizable.[3, 7, 8] Thus S_1, S_3, and S_7, which are known to be parallelizable, are the only spheres which have this property.

Let α: $\boldsymbol{SO}(n) \to \boldsymbol{U}(n)$ and β: $\boldsymbol{U}(n) \to \boldsymbol{SO}(2n)$ be the standard injections. α sends a matrix $A \in \boldsymbol{SO}(n)$ into itself, the entries of $\alpha(A)$ being regarded as complex numbers. β sends $C = (c_{u,v})$ into the $2n \times 2n$ matrix W, given by

$$w_{2u,2v} = w_{2u-1,2v-1} = a_{u,v}, \qquad w_{2u-1,2v} = -w_{2u,2v-1} = b_{u,v}$$

$(1 \leqq u, v \leqq n, c_{u,v} = a_{u,v} + ib_{u,v})$. We are interested in the induced homomorphisms on homotopy groups $\alpha_*:\pi_k(\boldsymbol{SO}(n)) \to \pi_k(\boldsymbol{U}(n))$, $\beta_*:\pi_k(\boldsymbol{U}(n)) \to \pi_k(\boldsymbol{SO}(2n))$,

and more particularly in the composition $\beta_*\alpha_*$: $\pi_k(\boldsymbol{SO}(n)) \to \pi_k(\boldsymbol{SO}(2n))$.

LEMMA 1. *In the stable range, i.e., for $k < n - 1$, one has $\beta_*\alpha_* = 2i_*$, where i_*: $\pi_k(\boldsymbol{SO}(n)) \to \pi_k(\boldsymbol{SO}(2n))$ is induced by the inclusion i: $\boldsymbol{SO}(n) \to \boldsymbol{SO}(2n)$.*

Since by R. Bott,[1] $\pi_{4s-1}(\boldsymbol{SO})$ and $\pi_{4s-1}(\boldsymbol{U})$ are infinite cyclic, the homomorphisms α_*: $\pi_{4s-1}(\boldsymbol{SO}(n)) \to \pi_{4s-1}(\boldsymbol{U}(n))$ and β_*: $\pi_{4s-1}(\boldsymbol{U}(n)) \to \pi_{4s-1}(\boldsymbol{SO}(2n))$ are represented (in the stable range, $4s + 1 \leqq n$) by multiplication with integers a_s, resp. b_s (which are determined only up to sign, since we do not specify our choice for the generators of $\pi_{4s-1}(\boldsymbol{SO})$ and $\pi_{4s-1}(\boldsymbol{U})$). From the above lemma follows the

COROLLARY: $a_s \cdot b_s = 2$.

It is not difficult to obtain $a_1 = 2$, $a_2 = 1$.

LEMMA 3. *a_s and b_s are periodic of period 2, i.e., $a_{s+2} = a_s$, $b_{s+2} = b_s$.* (This information is not needed for the theorem to be proved).

Consider now the commutative diagram in which n is to be large ($2s < n$):

$$\begin{array}{ccccc} \pi_{4s-1}(\boldsymbol{SO}(2n)) & \xrightarrow{p_*} & \pi_{4s-1}(V_{2n,2n-4s+2}) & \to & \pi_{4s-2}(\boldsymbol{SO}(4s-2)) \to 0 \\ \uparrow \beta_* & & \uparrow \beta'_* & & \uparrow \\ \pi_{4s-1}(\boldsymbol{U}(n)) & \xrightarrow{q_*} & \pi_{4s-1}(W_{n,n-2s+1}) & \to & \pi_{4s-2}(\boldsymbol{U}(2s-1)) \to 0, \end{array}$$

where the rows are portions of the homotopy sequences of the fibrations $\boldsymbol{SO}(2n)/\boldsymbol{SO}(4s-2) = V_{2n,2n-4s+2}$ with projection p, and $\boldsymbol{U}(n)/\boldsymbol{U}(2s-1) = W_{n,n-2s+1}$ with projection q, respectively. β': $\boldsymbol{U}(n)/\boldsymbol{U}(2s-1) \to \boldsymbol{SO}(2n)/\boldsymbol{SO}(4s-2)$ is induced by β: $\boldsymbol{U}(n) \to \boldsymbol{SO}(2n)$.

By R. Bott, $\pi_{4s-2}(\boldsymbol{U}(2s-1)) \approx \boldsymbol{Z}/(2s-1)!\boldsymbol{Z}$. Hence q_* is (up to sign) the multiplication by $(2s-1)!$. By B. Eckmann,[5] section 3.6, $\pi_{4s-1}(V_{2n,2n-4s+2}) \approx \boldsymbol{Z}_4$. For $s \geqq 3$, $(2s-1)!$ is divisible by 4, and thus ${\beta'}_*q_* = p_*\beta_* = 0$.

We need the following, probably well-known

LEMMA 2. *If S_{4s-1} is parallelizable, then $\pi_{4s-2}(\boldsymbol{SO}(4s-2)) = 0$.*

Let us assume now that S_{4s-1} is parallelizable. Then p_*: $\pi_{4s-1}(\boldsymbol{SO}(2n)) \to \pi_{4s-1}(V_{2n,2n-4s+2})$ is an epimorphism and hence maps a generator of $\pi_{4s-1}(\boldsymbol{SO}(2n))$ into a generator of $\pi_{4s-1}(V_{2n,2n-4s+2})$. Therefore, $p_*\beta_*\epsilon_U = b_s \cdot \epsilon_V$, where ϵ_U, ϵ_V are generators of $\pi_{4s-1}(\boldsymbol{U}(n))$, $\pi_{4s-1}(V_{2n,2n-4s+2})$, respectively. By Lemma 1, b_s is either 1 or 2 and hence $p_*\beta_* \neq 0$. Consequently, $s < 3$.

Proof of Lemma 1: Let A be the $2n \times 2n$ matrix

$$\begin{pmatrix} 0 & E \\ E & 0 \end{pmatrix},$$

i.e., $a_{ij} = \delta_{i+n,j}$ for $1 \leqq i \leqq n$ and $a_{ij} = \delta_{i-n,j}$ for $n < i \leqq 2n$. Since

$$i(X) = \begin{pmatrix} X & 0 \\ 0 & E \end{pmatrix},$$

we have

$$i(X) \cdot A \cdot i(X) \cdot A = \begin{pmatrix} X & 0 \\ 0 & X \end{pmatrix}.$$

Let M be the $2n \times 2n$ matrix given by

$$m_{ij} = \begin{cases} \delta_{2i-1,j} & \text{for} \quad 1 \leqq i \leqq n, \\ \delta_{2(i-n),j} & \text{for } n < i \leqq 2n. \end{cases}$$

It is easily verified that if $u_1, v_1, u_2, v_2, \ldots, u_n, v_n$ are the row vectors of a $2n \times 2n$ matrix Y, then the matrix $M \cdot Y$ has the row vectors $u_1, u_2, \ldots, u_n, v_1, v_2, \ldots, v_n$. This implies, using $(Y \cdot M')' = M \cdot Y'$ that if Y has column vectors $u'_1, v'_1, u'_2, v'_2, \ldots, u'_n, v'_n$, then $Y \cdot M'$ has the column vectors $u'_1, \ldots, u'_n, v'_1, \ldots, v'_n$. From this it follows that

$$\beta\alpha(X) = M \cdot i(X) \cdot A \cdot i(X) \cdot A \cdot M'$$

for any matrix $X \in \boldsymbol{SO}(n)$. Notice that $|A| = (-1)^n$ and $|M| = (-1)^{n(n-1)/2}$. Since we are interested in the stable range ($k - 1 < n$), there is no loss of generality in assuming n divisible by 4. Then $|A| = |M| = +1$. From the existence of paths from A and M to the unit $2n \times 2n$ matrix, follows: $\beta\alpha$ is homotopic to the map $\boldsymbol{SO}(n) \to \boldsymbol{SO}(2n)$, which sends X into $i(X) \cdot i(X) = i(X^2)$. By B. Eckmann,[4] Satz II, for any map $f: S_k \to \boldsymbol{SO}(n)$, the map f^2 given by $f^2(x) = [f(x)]^2$ represents $2\{f\}$, where $\{f\}$ is the homotopy class of f. This proves Lemma 1.

Proof of Lemma 2: By P. J. Hilton and J. H. C. Whitehead,[6] Lemma (4.12), if S_{4s-1} is parallelizable, then $\alpha_{4s-2} = \phi_*\mu$, where α_{4s-2} is the generator of $\pi_{4s-1}(S_{4s-2})$ the homomorphism ϕ_*: $\pi_{4s-1}(\boldsymbol{SO}(4s-1)) \to \pi_{4s-1}(S_{4s-2})$ is induced by the projection ϕ: $\boldsymbol{SO}(4s-1) \to S_{4s-2}$ and μ is some element in $\pi_{4s-1}(\boldsymbol{SO}(4s-1))$. From the homotopy sequence of $\boldsymbol{SO}(4s-1)/\boldsymbol{SO}(4s-2) = S_{4s-2}$, i.e.,

$$\ldots \to \pi_{4s-1}(\boldsymbol{SO}(4s-1)) \xrightarrow{\phi_*} \pi_{4s-1}(S_{4s-2}) \to \pi_{4s-2}(\boldsymbol{SO}(4s-2)) \to \pi_{4s-2}(\boldsymbol{SO}(4s-1)) \to 0,$$

and the fact that ϕ_* is an epimorphism, it follows that

$$\pi_{4s-2}(\boldsymbol{SO}(4s-2)) \approx \pi_{4s-2}(\boldsymbol{SO}(4s-1))$$

(if S_{4s-1} is parallelizable).

Consider the homotopy sequence of $\boldsymbol{SO}(4s)/\boldsymbol{SO}(4s-1) = S_{4s-1}$,

$$\ldots \to \pi_{4s-1}(\boldsymbol{SO}(4s)) \xrightarrow{\psi_*} \pi_{4s-1}(S_{4s-1}) \to \pi_{4s-2}(\boldsymbol{SO}(4s-1)) \to \pi_{4s-2}(\boldsymbol{SO}(4s)) = 0.$$

Since S_{4s-1} is assumed to be parallelizable, ψ_* is an epimorphism and therefore, $\pi_{4s-2}(\boldsymbol{SO}(4s-1)) = 0$. This proves Lemma 2.

Proof of Lemma 3: By formula (3.4) of R. Bott,[1] the space of loops over $\boldsymbol{SO}$ has the same homotopy groups as the quotient space $\boldsymbol{SO}/\boldsymbol{U}$. Thus for $2s < n$,

$$\pi_{4s-1}(\Omega\boldsymbol{SO}(2n)) \approx \pi_{4s-1}(\boldsymbol{SO}(2n)/\boldsymbol{U}(n)).$$

However, $\pi_{4s-1}(\Omega\boldsymbol{SO}(2n)) \approx \pi_{4s}(\boldsymbol{SO}(2n))$, this latter group being 0 for odd s and $\mathbf{Z}_2$ for s even. Since the order of $\pi_{4s-1}(\boldsymbol{SO}(2n)/\boldsymbol{U}(n))$ is clearly equal to b_s, we obtain: $b_s = 1$ or 2 according as to whether s is odd or even, respectively. The equality $a_s \cdot b_s = 2$ yields the result for a_s, which could also have been obtained directly using (3.3) of Bott.[1]

LEMMA 4. *For s odd $\geqq 3$, the generator of $\pi_{4s-1}(S_{4s-2})$ does not belong to Im ϕ_*, where ϕ_*: $\pi_{4s-1}(\boldsymbol{SO}(4s-1)) \to \pi_{4s-1}(S_{4s-2})$ is induced by the natural projection.*

Proof: We have seen that $s \geqq 3$ implies $p_*\beta_* = 0$. By Lemma 3, β_* is an epimorphism for s odd. Consequently, p_* must be trivial and $\pi_{4s-2}(\boldsymbol{SO}(4s-2)) \approx Z_4$. The exact homotopy sequence of the fibration $\boldsymbol{SO}(4s-1)/\boldsymbol{SO}(4s-2) = S_{4s-2}$ then yields the result.

The original version of this paper did not contain Lemmas 3 and 4. Lemma 3 was also observed by the referee. I understand from R. Bott that J. Milnor has also obtained our theorem.

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